

03/25/99 Jc586 U.S. PTO

PATENT  
674508-2001

FROMMER LAWRENCE & HAUG LLP  
745 FIFTH AVENUE  
NEW YORK, NEW YORK 10151  
TEL. (212) 588-0800

Date: March 25, 1999

Re: 674508-2001

ASSISTANT COMMISSIONER FOR PATENTS  
Box Patent Application (35 U.S.C. 111)  
Washington, D.C. 20231

Sir:

With reference to the filing in the United States Patent and Trademark Office of a Continuation application of a PCT application, pursuant to 35 U.S.C. 111(a) in the name of:

**FERSHT, Alan Roy; ZAHN, Ralph; and ALTAMIRANO, Myriam Marlenne**

entitled: **CHAPERONE FRAGMENTS**

X This is an application of a small entity under 37 CFR 1.9(f) and as only an unsigned Small Entity Verified Statement is enclosed, large entity fees have been paid, and a refund is respectfully requested upon the filing of the signed version of the Small Entity Verified Statement.

X Small Entity Verified Statement is enclosed.

The following are enclosed:

- X Specification (60 pages) and One Page of Abstract  
15 Sheet(s) of Drawings (Figs. 1 to 9e)  
X 50 Claim(s) (including 8 independent claim(s))  
This application contains a multiple dependent claim  
X Oath or Declaration and Power of Attorney signed X unsigned  
X Preliminary Amendment  
X Our check for \$845.00, calculated as follows:  
Basic Fee, \$760.00 (\$380.00)..... \$ 380.00  
Number of Claims in excess of 20 at \$18.00 (\$9.00) each: ..... 270.00  
Number of Independent Claims in excess of 3 at \$78.00 (\$39.00) each: ..... 195.00  
Multiple Dependent Claim Fee at \$260.00 (\$130.00)..... -0-  
Total Filing Fee ..... \$845.00  
Assignment Recording Fee \$40.00 ..... -0-  
X Pursuant to MPEP § 1895, enclosed is a copy of the PCT Request which is submitted as evidence that the instant continuation is copending with the PCT application and has at least one inventor in common therewith.  
— Certified copy of each of the following application(s) to substantiate the claim(s) for priority made in the Declaration:

<u>Application No.</u>	<u>Filed</u>	<u>In</u>
GB97/02652	26/6/97	PCT
GB96/02980	3/12/96	PCT
9620243.7	26/9/96	U.K.

Applicant or Patentee: FERSHT ET AL.  
Serial or Patent No.:  
Filed or Issued: March 25, 1999  
For: CHAPERONE FRAGMENTS

Frommer Lawrence & Haug LLP  
File No.: 674508-2001  
Page 1 of 2

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS**  
**(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name Of Organization: MEDICAL RESEARCH COUNSEL

Address Of Organization: 20 PARK CRESCENT  
LONDON W1N 4AL, GREAT BRITAIN

Type Of Organization:

- ☐ university or other institution of higher education  
☐ tax exempt under internal revenue service code (26 USC 501(a) and 502(c)(3))  
☐ nonprofit scientific or educational under statute of state of the United States of America  
☐ would qualify as tax exempt under internal revenue service code (26 USC 501(a) and 502(c)(3)) if located in the United States of America  
☒ would qualify as nonprofit scientific or educational under statute of state of the United States of America if located in the United States of America

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled **CHAPERONE FRAGMENTS** inventor(s): **Alan Roy FERSHT, Ralph ZAHN and Myriam Marlenne ALTAMIRANO** described in

- ☐ the specification filed herewith.  
☒ application serial no. , filed March 25, 1999.  
☐ patent no. , issued .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other

\*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27).

Applicant or Patentee: FERSHT ET AL.  
Serial or Patent No.:  
Filed or Issued: March 25, 1999  
For: CHAPERONE FRAGMENTS

**Frommer Lawrence & Haug LLP**  
File No.: 674508-2001  
Page 2 of 2

than the inventor who could not qualify as a small business concern under 35 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Full Name:

Address:

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Full Name:

Address:

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Full Name

Address

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing:

Title in Organization:

Address of Person Signing:

Signature: \_\_\_\_\_ Date: \_\_\_\_\_

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicant(s) : **FERSHT**, Alan, Roy; **ZAHN**, Ralph; **ALTAMIRANO**, Myriam,  
Marlenne

Serial No. : NOT YET ASSIGNED (CONTINUATION-IN-PART OF  
PCT/GB97/02652, FILED SEPTEMBER 26, 1997,  
AND DESIGNATING THE U.S., AND  
PUBLISHED APRIL 2, 1998 AS WO98/13496)

For : CHAPERONE FRAGMENTS

Filed : HEREWITH

Examiner : NOT YET ASSIGNED

Art Unit : NOT YET ASSIGNED

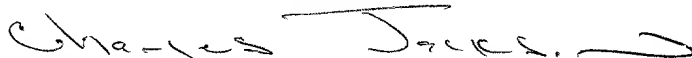
745 Fifth Avenue  
New York, NY 10151


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Date of Deposit: March 25, 1999

I hereby certify that this paper or fee is being deposited with the  
United States Postal Service "Express Mail Post Office to  
Addressee" Service under 37 CFR 1.10 on the date indicated  
above and is addressed to: Assistant Commissioner for Patents,  
Washington, DC 20231 Box New Patent Application

  
(Typed or printed name of person mailing paper or fee)

  
(Signature of person mailing paper or fee)

**PRELIMINARY AMENDMENT**

Assistant Commissioner for Patents  
Washington, D.C. 2023

Sir:

Prior to examination and fee calculation, and without any prejudice, admission, surrender  
of subject matter, or any intention of creating any estoppel as to equivalents, kindly amend the  
application as follows:



**IN THE SPECIFICATION**

Please amend the specification as follows:

Page 1, after the Title ("Chaperone Fragments") kindly insert:

**--RELATED APPLICATIONS**

This application is a continuation-in-part of PCT/GB97/02652, filed September 26, 1997, designating the U.S., claiming priority from PCT/GB96/02980, filed December 3, 1996 and GB 9620243.7, filed September 26, 1996; and, each of these documents, as well as all documents cited herein, and all documents referenced or cited in documents cited herein, are hereby incorporated herein by reference.

**FIELD OF THE INVENTION--.**

Page 1, between the first and second paragraphs (after "diagnostics." and before "Chaperones") please insert:

**--BACKGROUND OF THE INVENTION--.**

Page 7, between the first and second paragraphs (after "<10% homology)." and before "The present inventors ...") please insert:

**--OBJECTS AND SUMMARY OF THE INVENTION--.**

Page 7, third paragraph, please insert --a-- between "In" and "first".

Page 9, first paragraph, please insert --a-- between "In" and "second".

Page 9, first paragraph, please insert --a-- between "In" and "third".

Page 10, first full paragraph, please insert --a-- between "In" and "fourth".

Page 11, first full paragraph, please insert --a-- between "In" and "fifth".

Page 11, second full paragraph, please insert --a-- between "In" and "sixth".

Page 12, fourth paragraph, please insert --a-- between "In" and "seventh".

Page 13, third paragraph, please insert --an-- between “In” and “eighth” and please delete “as claimed in any preceding claim”.

Page 14, before the first full paragraph (before “A preferred polypeptide ...”) please insert:

--The term “...et seq ...” can have its ordinary meaning and thus the invention can include a polypeptide which comprises an amino acid sequence selected from GroEL residues between 228-273 and 194-328 such as: 227-273, 227-274, 226-274, 226-275, 225-275, 225-276, 224-276, 224-277, 223-277, 223-278, 222-278, 222-279, 221-279, 221-280, 220-280, 220-281, 219-281, 219-282, 218-282, 218-283, 217-283, 217-284, 216-284, 216-285, 215-285, 215-286, 214-286, 214-287, 213-287, 213-288, 212-288, 212-289, 211-289, 211-290, 210-290, 210-291, 209-291, 209-292, 208-292, 208-293, 207-293, 207-294, 206-294, 206-295, 205-295, 205-296, 204-296, 204-297, 203-297, 203-298, 202-298, 202-299, 201-299, 201-300, 200-300, 200-301, 199-301, 199-302, 198-302, 198-303, 197-303, 197-304, 196-304, 196-305, 195-305, 195-306, 194-306, 194-307, 195-328, 195-327, 196-327, 196-326, 197-326, 197-325, 198-325, 198-324, 199-324, 199-323, 200-323, 200-322, 201-321, 201-320, 202-320, 202-319, 203-319, 203-318, 204-318, 204-317, 205-317, 205-316, 206-316, 206-315, 207-315, 207-314, 208-314, 208-313, 209-313, 209-312, 210-312, 210-311, 211-311, 211-310, 212-310, 212-309, 213-309, 213-308, 214-308, 214-307, 215-307, 215-306, 216-306, 216-305, 217-305, 217-304, 218-304, 218-303, 219-303, 219-302, 220-302, 220-301, 221-301, 221-300, 222-300, 222-299, 223-299, 223-298, 224-298, 224-297, 225-297, 225-296, 226-296, 226-295, 227-295, 227-294, 221-303, 222-302, 223-301, 224-300, 225-299, 226-298, 227-297, 228-296, *inter alia*--

Page 14, second to last line, and page 15, line 1, after “preferably” and before “75%” (each occurrence), please insert: --at least--.

Page 15, after line 3 (“residues.”) and before line 4 (“The hsp60 class ...”), please insert:

--Of course, substantially homologous amino acid sequences and nucleotide sequences can have greater than 75% homology (e.g., at least 80% homology, or at least 85% homology, such as at least 90% homology, or even at least 95% homology, for instance at least 97% homology). Nucleotide sequence homology can be determined using the “Align” program of Myers and Miller, (“Optimal Alignments in Linear Space”, CABIOS 4, 11-17, 1988, incorporated herein by reference) and available at NCBI. Alternatively or additionally, the term “homology”, for instance, with respect to a nucleotide or amino acid sequence, can indicate a quantitative measure of homology between two sequences. The percent sequence homology can be calculated as  $(N_{ref} - N_{dif}) * 100 / N_{ref}$ , wherein  $N_{dif}$  is the total number of non-identical residues in the two sequences when aligned and wherein  $N_{ref}$  is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence similarity of 75% with the sequence AATCAATC ( $N_{ref} = 8$ ;  $N_{dif} = 2$ ). Alternatively or additionally, “homology” with respect to sequences can refer to the number of positions with identical nucleotides or amino acids divided by the number of nucleotides or amino acids in the shorter of the two sequences wherein alignment of the two sequences can be determined in accordance with the Wilbur and Lipman algorithm (Wilbur and Lipman, 1983 PNAS USA 80:726, incorporated herein by reference), for instance, using a window size of 20 nucleotides, a word length of 4 nucleotides, and a gap penalty of 4, and computer-assisted analysis and interpretation of the sequence data including alignment can be conveniently performed using commercially available programs (e.g., Intelligenetics™ Suite, Intelligenetics Inc. CA).. When RNA sequences are said to be similar, or have a degree of sequence identity or homology with DNA sequences, thymidine (T) in the DNA sequence is considered equal to uracil (U) in the RNA sequence.

RNA sequences within the scope of the invention can be derived from DNA sequences, by thymidine (T) in the DNA sequence being considered equal to uracil (U) in RNA sequences.

Additionally or alternatively, amino acid sequence similarity or identity or homology can be determined using the BlastP program (Altschul *et al.*, Nucl. Acids Res. 25, 3389-3402, incorporated herein by reference) and available at NCBI.. The following references (each incorporated herein by reference) provide algorithms for comparing the relative identity or homology of amino acid residues of two proteins, and additionally or alternatively with respect to the foregoing, the teachings in these references can be used for determining percent homology or identity: Needleman SB and Wunsch CD, "A general method applicable to the search for similarities in the amino acid sequences of two proteins," J. Mol. Biol. 48:444-453 (1970); Smith TF and Waterman MS, "Comparison of Bio-sequences," Advances in Applied Mathematics 2:482-489 (1981); Smith TF, Waterman MS and Sadler JR, "Statistical characterization of nucleic acid sequence functional domains," Nucleic Acids Res., 11:2205-2220 (1983); Feng DF and Dolittle RF, "Progressive sequence alignment as a prerequisite to correct phylogenetic trees," J. of Molec. Evol., 25:351-360 (1987); Higgins DG and Sharp PM, "Fast and sensitive multiple sequence alignment on a microcomputer," CABIOS, 5: 151-153 (1989); Thompson JD, Higgins DG and Gibson TJ, "ClusterW: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, positions-specific gap penalties and weight matrix choice," Nucleic Acid Res., 22:4673-480 (1994); and, Devereux J, Haeberlie P and Smithies O, "A comprehensive set of sequence analysis program for the VAX," Nucl. Acids Res., 12: 387-395 (1984).

Likewise, from the text herein, one skilled in the art can determine what is meant by “homology” and “substantially homologous” without any undue experimentation, such that these terms are clear and definite (note for instance the following text).--.

Page 22, first full paragraph, please change “The polypeptide” to --In a ninth aspect, the invention provides a polypeptide which--.

Page 24, third paragraph, please insert --a-- between “In” and “tenth”.

Page 26, second full paragraph, please insert --an-- between “In” and “eleventh”.

Page 26, third full paragraph, please insert --a-- between “In” and “twelfth”.

Page 26, last paragraph, please insert --a-- between “In” and “thirteenth”.

Page 27, first full paragraph, please insert --a-- between “In” and “fourteenth”.

Page 31, second full paragraph, please insert --a-- between “In” and “fifteenth”.

Page 31, the paragraph spanning pages 31-32, please insert --a-- between “In” and “sixteenth”.

Page 32, first full paragraph, please insert --a-- between “In” and “seventeenth”.

Page 32, at the end of the second full paragraph (after “about 1:1”) please insert: --And, this can be an eighteenth aspect of the invention.--

Page 32, third full paragraph, please insert --a-- between “In” and “nineteenth”.

Page 32, fourth full paragraph, please insert --a-- between “In” and “twentieth”--.

Page 32, last (fifth full) paragraph, please insert --a-- between “In” and “twenty-first”.

Page 33, first full paragraph, please insert --a-- between “In” and “twenty-second”.

Page 33, second full paragraph, please insert --a-- between “In” and “twenty-third”.

Page 33, between the second and third full paragraphs (after “antagonist thereof” and before “The monomeric apical domain”) please insert:

--A construct encoding a polypeptide of the invention or an antagonist thereof would be a vector or a recombinant for expression of the polypeptide or antagonist. The methods for making and/or administering a vector or recombinant for expression of such agents either *in vivo* or *in vitro* can be any desired method, e.g., a method which is by or analogous to the methods disclosed in: U.S. Patent Nos. 4,603,112, 4,769,330, 5,174,993, 5,505,941, 5,338,683, 5,494,807, 4,722,848, WO 94/16716, WO 96/39491, Paoletti, "Applications of pox virus vectors to vaccination: An update," PNAS USA 93:11349-11353, October 1996, Moss, "Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety," PNAS USA 93:11341-11348, October 1996, Smith et al., U.S. Patent No. 4,745,051 (recombinant baculovirus), Richardson, C.D. (Editor), Methods in Molecular Biology 39, "Baculovirus Expression Protocols" (1995 Humana Press Inc.), Smith et al., "Production of Human Beta Interferon in Insect Cells Infected with a Baculovirus Expression Vector," Molecular and Cellular Biology, Dec., 1983, Vol. 3, No. 12, p. 2156-2165; Pennock et al., "Strong and Regulated Expression of *Escherichia coli* B-Galactosidase in Infect Cells with a Baculovirus vector," Molecular and Cellular Biology Mar. 1984, Vol. 4, No. 3, p. 399-406; EPA 0 370 573, U.S. application Serial No. 920,197, filed October 16, 1986, EP Patent publication No. 265785, U.S. Patent No. 4,769,331 (recombinant herpesvirus), Roizman, "The function of herpes simplex virus genes: A primer for genetic engineering of novel vectors," PNAS USA 93:11307-11312, October 1996, Andreansky et al., "The application of genetically engineered herpes simplex viruses to the treatment of experimental brain tumors," PNAS USA 93:11313-11318, October 1996, Robertson et al. "Epstein-Barr virus vectors for gene delivery to B lymphocytes," PNAS USA 93:11334-11340, October 1996, Frolov et al., "Alphavirus-based expression vectors: Strategies and applications," PNAS USA 93:11371-11377, October 1996, Kitson et al., J. Virol.

65, 3068-3075, 1991; U.S. Patent Nos. 5,591,439, 5,552,143 (recombinant adenovirus), Grunhaus et al., 1992, "Adenovirus as cloning vectors," Seminars in Virology (Vol. 3) p. 237-52, 1993, Ballay et al. EMBO Journal, vol. 4, p. 3861-65, Graham, Tibtech 8, 85-87, April, 1990, Prevec et al., J. Gen Virol. 70, 429-434, PCT WO91/11525, Felgner et al. (1994), J. Biol. Chem. 269, 2550-2561, Science, 259:1745-49, 1993 and McClements et al., "Immunization with DNA vaccines encoding glycoprotein D or glycoprotein B, alone or in combination, induces protective immunity in animal models of herpes simplex virus-2 disease," PNAS USA 93:11414-11420, October 1996, and U.S. Patents Nos 5,591,639, 5,589,466, and 5,580,859 relating to DNA expression vectors, *inter alia*. See also WO 98/33510; Ju et al., Diabetologia, 41:736-739, 1998 (lentiviral expression system); Sanford et al., U.S. Patent No. 4,945,050 (method for transporting substances into living cells and tissues and apparatus therefor); Fischbach et al. (Intracel), WO 90/01543 (method for the genetic expression of heterologous proteins by cells transfected); Robinson et al., seminars in IMMUNOLOGY, vol. 9, pp.271-283 (1997) (DNA vaccines); Szoka et al., U.S. Patent No. 4,394,448 (method of inserting DNA into living cells); and McCormick et al., U.S. Patent No. 5,677,178 (use of cytopathic viruses for therapy and prophylaxis of neoplasia).

The expression product generated by vectors or recombinants in this invention optionally can also be isolated and/or purified from infected or transfected cells; for instance, to prepare compositions for administration to patients. However, in certain instances, it may be advantageous to not isolate and/or purify an expression product from a cell; for instance, when the cell or portions thereof enhance the effect of the polypeptide or the antagonist thereof.

More generally, compositions for use in the invention, e.g., compositions containing antibodies or polypeptides or antagonists or vectors or recombinants, can be prepared in

accordance with standard techniques well known to those skilled in the pharmaceutical or medical arts. Such compositions can be administered in dosages and by techniques well known to those skilled in the medical arts taking into consideration such factors as the age, sex, weight, and condition of the particular patient, and the route of administration and the condition or disease being treated. The compositions can be administered alone, or can be co-administered or sequentially administered with other compositions of the invention or with other prophylactic or therapeutic compositions.

Examples of compositions of the invention include liquid preparations for orifice, e.g., oral, nasal, anal, genital (e.g., vaginal), vascular and/or SMC, etc., administration such as suspensions, syrups or elixirs; and, preparations for parenteral, subcutaneous, intradermal, intramuscular, intravenous, intraarterial, intralymphatic, or intraperitoneal administration (e.g., injectable administration) such as sterile suspensions or emulsions. In such compositions the active agent can be in admixture with a suitable carrier, diluent, or excipient such as sterile water, physiological saline, glucose or the like.

The compositions of the invention may be packaged in a single dosage form for immunization by parenteral (i.e., intramuscular, intradermal or subcutaneous) administration or orifice administration, e.g., perlingual (i.e., oral), intragastric, mucosal including intraoral, intraanal, intravaginal, intravenous, intralymphatic, intraarterial, intraperitoneal, and the like administration. Accordingly, compositions in forms for such administration routes are envisioned by the invention. And again, the effective dosage and route of administration are determined by known factors, such as age, sex, weight, condition and nature of patient disease or condition being treated, as well as LD<sub>50</sub> and other screening procedures which are known and do not require undue experimentation.



Dosages of each active agent can range from a few to a few hundred micrograms, e.g., 5 to 500  $\mu$ g. An inventive vector or recombinant expressing a polypeptide and/or an antagonist thereof can be administered in any suitable amount to achieve expression at these dosage levels. The inventive vector or recombinant can be administered to a patient or infected or transfected into cells in an amount of about at least  $10^3$  pfu; more preferably about  $10^4$  pfu to about  $10^{10}$  pfu, e.g., about  $10^5$  pfu to about  $10^9$  pfu, for instance about  $10^6$  pfu to about  $10^8$  pfu. And, if more than one gene product is expressed by more than one recombinant, each recombinant can be administered in these amounts; or, each recombinant can be administered such that there is, in combination, a sum of recombinants comprising these amounts. Other suitable carriers or diluents can be water or a buffered saline, with or without a preservative. The expression product or isolated product or vector or recombinant may be lyophilized for resuspension at the time of administration or can be in solution. Antibodies can be humanized to enhance their effects. See, e.g., Huls et al., "A recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments," Nature Biotechnology Vol. 17, No. 3, March 1999, and documents cited therein, incorporated herein by reference.

In plasmid compositions, the dosage should be a sufficient amount of plasmid to elicit a response analogous to compositions wherein the agent or agents are directly present; or to have expression analogous to dosages in such compositions; or to have expression analogous to expression obtained *in vivo* by recombinant compositions. For instance, suitable quantities of plasmid DNA in plasmid compositions can be 1  $\mu$ g to 100 mg, preferably 0.1 to 10 mg, e.g., 500 micrograms, but lower levels such as 0.1 to 2 mg or preferably 1-10  $\mu$ g may be employed. Documents cited herein regarding DNA plasmid vectors may be consulted for the skilled artisan

to ascertain other suitable dosages for DNA plasmid vector compositions of the invention, without undue experimentation.

For treatment of a disease, the compositions comprising the polypeptide and/or the antagonist thereof, alone or with other treatment, may be administered as desired by the skilled medical practitioner, from this disclosure and knowledge in the art, e.g., at the first signs or symptoms, or as soon thereafter as desired by the skilled medical practitioner, without any undue experimentation required; and, the administration of the compositions, alone or with other treatment, may be continued as a regimen, e.g., monthly, bi-monthly, biannually, annually, or in some other regimen, by the skilled medical practitioner for such time as is necessary to prevent or treat the disease, without any undue experimentation required.

For prevention of a disease, the compositions, alone or with other treatment, may be administered at the first indication of the patient being prone to the disease, or as soon thereafter as desired by the skilled medical practitioner, in any desired regimen, such as a single administration or multiple administrations in a regimen as desired, e.g., monthly, bi-monthly, biannually, or any combination thereof, or in some other regimen, by the skilled medical practitioner for such time as is necessary to prevent the disease, without any undue experimentation required.--

Page 37, before the first line, please insert:

**--BRIEF DESCRIPTION OF THE DRAWINGS--**.

Page 39, after the fourth full paragraph (after line 8, actual count, i.e., after “for comparison.”) and before “Example 1” (before line 9, actual count), please insert:\

**--DETAILED DESCRIPTION**

**EXAMPLES--**.

**IN THE CLAIMS**

Please amend the claims, without any prejudice, without any surrender of subject matter, without any intention of creating any estoppel as to equivalents, and without any admission, as follows:

Claim 9, line 1, please change "in any preceding claim" to --claim 1--.

Claim 11, line 1, please change "in any preceding claim" to --claim 1--; and, in line 2, please change "preferably" to --or--.

Claim 12, line 1, please delete "as claimed in any preceding claim".

Claim 14, line 1, please change "in any preceding claim" to --claim 1--; and, in line 2, please change "preferably" to --or--.

Claim 16, line 1, please delete "or claim 15"; and, in line 2, please change "preferably" to --or--.

Claim 17, line 1, please delete "in any one of", change "claims" to --claim--, delete "to 16"; and, in line 3, please change "preferably" (each occurrence) to --or--.

Claim 18, line 1, please change "in any preceding claim" to --claim 1--, and "and is" to --in--; and, in line 2, please change "preferably" to --or--.

Claim 20, line 1, please change "in any preceding claim" to --claim 1--.

Claim 21, line 1, please change "in any preceding claim" to --claim 1--.

Claim 22, line 2, please delete "any one of" and "to 21" and change "claims" to --claim--.

Claim 24, line 1, please delete "claim 22 or".

Claim 25, lines 1 and 2, please delete "claim 22 or" and "or vector as defined in claim 24".

Claim 26, line 1, please delete "any one of" and "to 21" and change "claims" to --claim--.

27. (Amended) A method of making a polypeptide [as claimed in claim 26]  
comprising transforming a host cell with a nucleic acid encoding said polypeptide and culturing  
the transformed cell and expressing said polypeptide wherein the nucleic acid is as defined in  
[claim 22 or] claim 23.

Claim 28, line 1, please delete “claim 26 or”.

Claim 29, lines 1-2, please delete “any one of” and “to 21” and change “claims” to  
--claim--.

Claim 30, line 1, please delete “any one of” and “to 21” and change “claims” to --claim--.

Claim 31, line 1, please delete “any one of” and “to 21” and change “claims” to --claim--.

Claim 32, line 1, please delete “claims 22 or”.

Claim 33, line 1, please delete “claim 22 or”.

Claim 34, line 2, please delete “any one of” and “to 21” and change “claims” to --claim--.

Claim 36, line 1, please delete “claim 34 or”.

Claim 37, line 1, please delete “claim 33 or”

Claim 38, line 1, please delete “any one of” and “to 21” and change “claims” to --claim--.

Claim 40, line 1, please delete “claim 38 or”.

Claim 41, line 1, please delete “any one of” and “to 21” and change “claims” to --claim--.

Claim 42, line 2, please delete “any one of” and “to 21” and change “claims” to --claim--.

Claim 43, line 1, please delete “any one of” and “to 21” and change “claims” to --claim--.

Claim 44, lines 1-2, please delete “any one of” and “to 21” and change “claims” to  
--claim--.

Claim 47, line 2, please delete “any one of” and “to 21” and change “claims” to --claim--.

Claim 48, lines 2-3, please delete “any one of” and “to 21” and change “claims” to

--claim--.

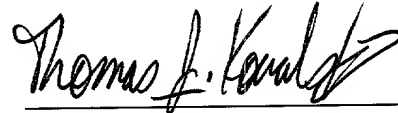
Claim 50, line 2, please delete "any one of" and "to 21" and change "claims" to --claim--.

**REMARKS**

This Amendment places the specification and claims into better form, removes multiple dependencies, and adds a lineage to the predecessor PCT and UK applications, without any prejudice, admission, intention of creating any estoppel as to equivalents, and without any surrender of subject matter. Early and favorable examination on the merits is earnestly solicited.

Respectfully submitted,  
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## Chaperone Fragments

The present invention relates to chaperone polypeptides which are active in the  
5 folding and maintenance of structural integrity of other proteins. The invention also  
relates to nucleic acids encoding chaperone polypeptides, vectors comprising these  
nucleic acids, host cells modified with the nucleic acids or vectors so as to express  
the chaperone polypeptides. The invention further relates to methods of making  
chaperone polypeptides whether by synthetic or recombinant means, pharmaceutical  
10 compositions comprising the chaperone polypeptides or nucleic acids encoding the  
same and the use of chaperone polypeptides in the treatment of disease or in the  
reconditioning of biologically active materials. The invention also relates to  
antibodies reactive against chaperone polypeptides and their use in medicine and  
diagnostics.

15 Chaperones are in general known to be large multisubunit protein assemblies  
essential in mediating polypeptide chain folding in a variety of cellular  
compartments. Families of chaperones have been identified, for example the  
chaperonin hsp60 family otherwise known as the cpn60 class of proteins are  
20 expressed constitutively and there are examples to be found in the bacterial  
cytoplasm (GroEL), in endosymbiotically derived mitochondria (hsp60) and in  
chloroplasts (Rubisco binding protein). Another chaperone family is designated  
TF55/TCP1 and found in the thermophilic archaea and the evolutionarily connected  
eukaryotic cytosol. A comparison of amino acid sequence data has shown that there  
25 is at least 50% sequence identity between chaperones found in prokaryotes,  
mitochondria and chloroplasts (Ellis R J and Van der Vies S M (1991) Ann Rev  
Biochem 60: 321-347).

A typical chaperonin is GroEL which is a member of the hsp60 family of heat shock  
30 proteins. GroEL is a tetradecamer wherein each monomeric subunit (cpn60m) has a

molecular weight of approximately 57kD. The tetradecamer facilitates the *in vitro* folding of a number of proteins which would otherwise misfold or aggregate and precipitate. The structure of GroEL from *E. coli* has been established through X-ray crystallographic studies as reported by Braig K *et al* (1994) Nature 371: 578-586. The holo protein is cylindrical, consisting of two seven-membered rings that form a large central cavity which according to Ellis R J and Hartl F U (1996) FASEB Journal 10: 20-26 is generally considered to be essential for activity. Some small proteins have been demonstrated to fold from their denatured states when bound to GroEL (Gray T E and Fersht A R (1993) J Mol Biol 232: 1197-1207; Hunt J F *et al* (1996) Nature 379: 37-45; Weissman J S *et al* (1996) Cell 84: 481-490; Mayhew M *et al* (1996) Nature 379: 420-426; Corrales F J and Fersht A R (1995) Proc Nat Acad Sci 92: 5326-5330) and it has been argued that a cage-like structure is necessary to sequester partly folded or assembled proteins (Ellis R J and Hartl F U (1996) *supra*.

The entire amino acid sequence of *E. coli* GroEL is also known (see Braig K *et al* (1994) *supra*) and three domains have been ascribed to each cpn60m of the holo chaperonin (tetradecamer). These are the intermediate (amino acid residues 1-5, 134-190, 377-408 and 524-548), equatorial (residues 6-133 and 409-523) and apical (residues 191-376) domains.

Monomers of GroEL have been induced by urea or pressure, but they are inactive and have to reassociate to form the central cavity in order to facilitate the refolding of rhodanese (Mendoza J A *et al* (1994) J Biol Chem 269: 2447-2451; Ybarra J and Horowitz P M (1995) J Biol Chem 270: 22962-22967).

GroEL facilitates the folding of a number of proteins by two mechanisms; (1) it prevents aggregation by binding to partly folded proteins (Goloubinoff P *et al* (1989) Nature 342: 884-889; Zahn R and Plückthun A (1992) Biochemistry 31: 3249-3255), which then refold on GroEL to a native-like state (Zahn R and

- Plückthun A (1992) *Biochemistry* **31**: 3249-3255; Gray T E and Fersht A R (1993) *J Mol Biol* **232**: 1197-1207; and (2) it continuously anneals misfolded proteins by unfolding them to a state from which refolding can start again (Zahn R *et al* (1996) *Science* **271**: 642-645). Some mutations in the apical domain led to a decrease in polypeptide binding (Fenton W A *et al* (1994) *Nature* **371**: 614-619), suggesting that this domain is involved in the binding of polypeptides. Electron microscopy suggests that denatured protein binds to the inner side of the apical end of the GroEL-cylinder (Chen S *et al* (1994) *Nature* **371**: 261-264). The equatorial domain has been shown from the 2.4 Å crystal structure of ATP $\gamma$ S-ligated GroEL (Boisvert D C *et al* (1996) *Nature Structure Biology* **3**: 170-177) and mutagenesis studies (Fenton W A *et al* (1994) *Nature* **371**: 614-619) to have the nucleotide binding sites. Binding and hydrolysis of ATP is cooperative (Bochkareva E S *et al* (1992) *J Biol Chem* **267**: 6796-6800; Gray T E and Fersht A R (1991) *FEBS Lett* **292**: 254-258), and lowers the affinity for polypeptides (Jackson G S *et al* (1993) *Biochemistry* **32**: 2554-2563). Most of the intermolecular contacts between the subunits of GroEL are between the equatorial domain. The intermediate domain connects the other two domains, transmitting allosteric effects (Braig K *et al* (1994) *Nature* **371**: 578-586; Braig K *et al* (1995) *Nature Struct Biol* **2**: 1083-1094).
- 20 The crystal structure of GroEL shows unusually high *B-factors* for the apical domain compared with the equatorial or intermediate domain, and the *B-factors* vary considerably within the domain (Braig K *et al* (1994) *Nature* **371**: 578-586; Braig K *et al* (1995) *Nature Struct Biol* **2**: 1083-1094; Boisvert D C *et al* (1996) *Nature Structure Biology* **3**: 170-177). The high overall *B-factor* seems to result from a static disorder within the asymmetric unit and probably throughout the crystals of GroEL, and has been attributed to rigid-body movements generated by hinge-like  $\beta$ -sheets in the intermediate domain. Regions of high flexibility have also been observed in the 2.8Å structure of the co-chaperonin GroES (Hunt J F *et al* (1996) *Nature* **379**: 37-45). A mobile loop has been shown to be directly involved in ADP-dependent binding to the apical domain (Landry S J *et al* (1993) *Nature*



364: 255-258). Binding of GroES leads to a conformational change of GroEL and a concomitant enlargement of the GroEL-cavity (Chen S *et al* (1994) Nature 371: 261-264), in which the encapsulated polypeptide substrate can refold to a native-like state without the danger of aggregation (Martin J *et al* (1993) Nature 366: 228-233; Weissman J S *et al* (1995) Cell 83: 577-587).

Monomeric forms of GroEL have been induced by site-directed mutagenesis and expressed and although these bind to rhodanese they do not affect its refolding (White Z W *et al* (1995) J Biol Chem 270: 20404-20409).

Yoshida *et al* (1993) FEBS 336: 363-367 report that a 34kD proteolytic fragment of *E. coli* GroEL which lacks 149 NH<sub>2</sub>-terminal residues and ~93 COOH-terminal residues (GroEL 150-456) facilitates refolding of denatured rhodanese in the absence of GroES and ATP. Although the proteolytic fragment GroEL 150-456 elutes as a monomer during gel filtration, it still comprises the apical domain and significant portions of the intermediate and equatorial domains, the latter of which determine the intersubunit contacts of GroEL (Braig K *et al* (1994) *supra*), thus allowing transient formation of the central cavity thereby accounting for the chaperonin activity which is observed.

In any event, the mode of rhodanese refolding by GroEL 150-456 is very different from that brought about by the holo protein; the yield of productive refolding is low, folding is rapidly saturated with time, and it is not affected by GroES and ATP. Efficient release and folding requires the hydrolysis of ATP (Landry S J *et al* (1992) Nature 355: 455-457; Gray T E and Fersht A R (1992) FEBS Lett 282: 254-258; Jackson G S *et al* (1993) Biochemistry 32: 2554-2563; Todd M *et al* (1993) Biochemistry 32: 8560-8567.)

EP-A-0 650 975 (NIPPON OIL CO LTD) discloses chaperonin molecules and a method of refolding denatured proteins using GroEL chaperonin 60 monomers

(cpn60m) obtained from *Thermus thermophilus*. The holo-chaperonin was first extracted and then purified from the bacterial source according to the method of Taguchi *et al* (1991) J Biol Chem 266: 22411-22418. The cpn60m was then produced by treatment of the holo-chaperonin with trifluoroacetic acid (TFA) followed by reverse phase (rp) HPLC of the resulting denatured protein. A peak fraction containing the approximately 57kD cpn60m was obtained. The refolding activity of the cpn60m was assayed in solution by monitoring the regain in activity of inactivated rhodanese, which in specific activity terms amounted to about only 25% of the specific activity of the rhodanese prior to inactivation. When background spontaneous rhodanese refolding is subtracted then there is only an approximately 20% refolding activity.

As well as cpn60m, EP-A-0 650 975 also discloses the use of an approximately 50kD N-terminal deletion fragment of cpn60m wherein the N-terminal amino acid residues up to (but not including) the Thr residue at position 79 are removed by proteolysis. This 50kD fragment showed an approximately 35% (about 30% when background is subtracted) rhodanese refolding activity when in solution.

Taguchi H *et al* (1994) J Biol Chem 269: 8529-8534 is a scientific report on which the invention of EP-A-0 650 975 is based. A transiently formed GroEL tetradecamer (the holo-chaperonin) was perceived to exist when the chaperonin monomers are present in solution. Consequently, the refolding activity of these preparations can be seen to be caused by the presence of holo chaperonin, not monomers. To test this, Taguchi *et al* immobilised cpn60m to a chromatographic resin to exclude the possibility of holo chaperonin formation. When immobilised and therefore when in truly monomeric form, cpn60m exhibited only about 10% rhodanese refolding activity.

The refolding of rhodanese has been a common and convenient assay to determine chaperonin activity but it has been observed that there are significant problems with

the assay which cast serious doubt on existing assertions of refolding activity based on this assay. The fact is that rhodanese refolds spontaneously in the absence of molecular chaperones with the yield of refolded rhodanese increasing progressively as the rhodanese concentration decreases (see Taguchi *et al* (1994) *supra*). The  
5 10% of rhodanese refolding activity reported in EP-A-0 650 975 for immobilised (truly monomeric) cpn60m is therefore too close to the spontaneous regain of activity by rhodanese to demonstrate that any monomeric chaperonin has a refolding activity towards proteins generally, let alone cpn60m and rhodanese.

10 Alconada A and Cuezva J M (1993) TIBS 18: 81-82 suggested that an "*internal fragment*" of GroEL may possess a chaperone activity on the basis of amino acid sequence similarity between the altered mRNA stability (ams) gene product (Ams) of *E. coli* and the central part of GroEL. The ams locus is a temperature-sensitive mutation that maps at 23 min on the *E. coli* chromosome and results in mRNA with  
15 an increased half-life. The ams gene has been cloned, expressed and shown to complement the ams mutation. The gene product is a 149-amino acid protein (Ams) with an apparent molecular weight of 17kD.

Chanda P K *et al* (1985) J Bacteriol 161: 446-449 found that a 17kD protein  
20 fragment corresponding to part of the L gene of the groE operon, when expressed in *E. coli* ams mutants restores the wild-type phenotype. This 17kD fragment was suggested as being an isolated, functional chaperonin protein module. The amino acid sequences of three chaperonins (*E. coli* GroEL, ribulose biphosphate carboxylase (RUBPC) subunit-binding protein from *Triticum aestivum* and  
25 *Saccharomyces cerevisiae* mitochondrial hsp60) were compared with the sequence of Ams. Residues 307-423 were found to correspond substantially between Ams and GroEL. These residues comprise nearly equivalent portions of both the intermediate and apical domains of GroEL.

The sequence alignments of Ams protein with the chaperonins noted above reveals a striking similarity (98%) between the amino-terminal four-fifths of Ams and the central part (approximately one-fifth) of *E. coli* GroEL chaperonin. The 50% sequence similarity between the Ams amino terminal region and the two other  
5 chaperonins is in line with the reported identity among the chaperonin family. The carboxy-terminal part of the Ams protein showed no similarity with chaperonins (<10% homology).

The present inventors have identified a need for a simple, truly monomeric  
10 chaperone molecule which is of defined sequence and structure and which can efficiently and reproducibly refold, renature, reactivate or recondition proteins from a range of sources in the absence of added cofactor or other agents and without the need to associate to form a holo chaperonin. A problem which the invention seeks to solve is therefore how to provide an active portion or fragment of  
15 a chaperone in truly monomeric form so as to promote a useful and efficient reagent for the refolding, renaturing or reconditioning of biological molecules, particularly proteins. A further object is the provision of such a monomeric form at minimum size.

20 In first aspect the present invention provides a chaperone polypeptide having an amino acid sequence selected from at least amino acid residues 230-271 but no more than residues 150-455 or 151-456 of a GroEL sequence substantially as shown in Figure 7, or a corresponding sequence of a substantially homologous chaperone polypeptide, or a modified, mutated or variant thereof having chaperone activity.

25

The sequence of GroEL is available in the art, as set forth above, and from academic databases; however, GroEL fragments which conform to the database sequence are inoperative. Specifically, the database contains a sequence in which positions 262 and 267 are occupied by Alanine and Isoleucine respectively.  
30 Fragments incorporating one or both of these residues at these positions are

inoperative and unable to promote the folding of polypeptides. The invention, instead, relates to a GroEL polypeptide in which at least one of positions 262 and 267 is occupied by Leucine and Methionine respectively.

- 5 The amino acid sequence is preferably selected from at least amino acid residues 193-335, preferably 193-337, more preferably 191-345, even more preferably 191-376 but no more than residues 151-455. The invention therefore includes polypeptides being GroEL amino acid residues 230-271, 230-272 ...*et seq.* 230-455 and in like manner residues 230-271, 229-271 ...*et seq.* 151-271. Also,
- 10 residues 230-271, 229-272 ...*et seq.* 151-351, 151-352 ...*et seq.* 151-455. All amino acid sequences of 42 or more residues comprising at least contiguous residues 230-271 and not exceeding 151-455 are within the scope of this aspect of the invention eg 171-423 or 166-406.
- 15 In a highly preferred aspect, the invention provides fragments selected from the group consisting of residues 191-375, 191-345 and 193-335.

There are four key properties that may characterise a protein as a molecular chaperone (1) suppression of aggregation during protein folding; (2) suppression of

20 aggregation during protein unfolding; (3) influence on the yield and kinetics of folding; and (4) effects exerted at near stoichiometric levels.

Chaperone activity may be determined in practice by an ability to refold cyclophilin A but other suitable proteins such as glucosamine-6-phosphate deaminase or a

25 mutant form of indoleglycerol phosphate synthase (IGPS) (amino acid residues 49-252) may be used. A rhodanese refolding assay may also be used. Details of suitable refolding assays are described in more detail in the specific examples provided hereinafter.

In second aspect the invention provides monomeric polypeptide having chaperone activity and incapable of multimerisation in solution.

In third aspect the invention provides a chaperone polypeptide which, when in solution, remains monomeric and has the ability to refold, reactivate or recondition proteins, said polypeptide including the protein binding active site motif:

1 X X X 2 X X 3 4 X X 5 X X X X X X X X X X X X X X 6 X X 7  
8 X 9 10 11 X X 12 X 13 14

10

wherein 1 is selected from amino acid residues:

I, M, L, V, S, F or A;

wherein 2 is selected from: L, I, P, V or A;

wherein 3 is selected from: L, E, V, H or I;

15 wherein 4 is selected from: E, A, R, L, Q, or N;

wherein 5 is selected from: A, V, I, M, L, N, S, R, T, Q or K;

wherein 6 is selected from: E, D or G;

wherein 7 is selected from: A, P, S, T, G or L;

wherein 8 is selected from: T, A, N, S or V;

20 wherein 9 is selected from: V, L, I or A;

wherein 10 is selected from: V, L, I, F or H;

wherein 11 is selected from: N, S or L;

wherein 12 is selected from: R, K, N, Q, L or S;

wherein 13 is selected from: I, T, S, G, V, A, Q, N, K, F or P;

25 wherein 14 is selected from: V, I, L, F, D or T; and

wherein the X's represent a peptide bond or bonds or at least one amino acid residue,

or a functional variant thereof in which one or more of the numbered amino acid residues 1 to 14 has undergone a conservative substitution.

In fourth aspect the invention provides a chaperone polypeptide which, when in solution, remains monomeric and has the ability to refold, reactivate or recondition proteins, said polypeptide including at least one protein binding active site motif moiety selected from:

(a) 1 X X X 2 X X 3 4 X and

(b) X 5 X X X X X X X X X X X X X 6 X X 7 8 X  
9 10 11 X X 12 X 13 14

wherein 1 is selected from amino acid residues:

I, M, L, V, S, F or A;

wherein 2 is selected from: L, I, P, V or A;

wherein 3 is selected from: L, E, V, H or I;

wherein 4 is selected from: E, A, R, L, Q, or N;

wherein 5 is selected from: A, V, I, M, L, N, S, R, T, Q or K;

wherein 6 is selected from: E, D or G;

wherein 7 is selected from: A, P, S, T, G or L;

wherein 8 is selected from: T, A, N, S or V;

wherein 9 is selected from: V, L, I or A;

wherein 10 is selected from: V, L, I, F or H;

wherein 11 is selected from: N, S or L;

wherein 12 is selected from: R, K, N, Q, L or S;

wherein 13 is selected from: I, T, S, G, V, A, Q, N, K, F or P;

wherein 14 is selected from: V, I, L, F, D or T; and

wherein X is at least one amino acid residue,

or a functional variant thereof in which one or more of the numbered amino acid residues 1 to 14 has undergone a conservative substitution.

- 5 In fifth aspect the claim provides a chaperone polypeptide which, when in solution, remains monomeric and has the ability to refold, reactivate or recondition proteins, said polypeptide including the protein binding active site motif:

10 I X X X L X X L E X X A X X X X X X X X X X X X X X E X X  
A T X V V N X X R X I V

wherein X is at least one amino acid residue, or a functional variant thereof in which one or more of the specified amino acid residues has undergone a conservative substitution.

- 15 In sixth aspect the claims provides a chaperone polypeptide which, when in solution, remains monomeric and has the ability to refold, reactivate or recondition proteins, said polypeptide including at least one protein binding active site motif moiety selected from:

20

(a) I X X X L X X L E X

(b) X A X X X X X X X X X X X X X X X E X X A T X  
V V N X X R X I V

25

wherein X is at least one amino acid residue, or a functional variant thereof in which one or more of the specified amino acid residues has undergone a conservative substitution.



A conservative substitution is the replacement of one amino acid residue for another chemically or functionally similar amino acid residue such that the function of the polypeptide overall remains substantially unchanged.

- 5 The terms "refold", "reactivate" and "recondition" are not intended as being mutually exclusive. For example, an inactive protein, perhaps denatured using urea may have an unfolded structure. This inactive protein may then be refolded with a polypeptide of the invention thereby reactivating it. In some circumstances there may be an increase in the specific activity of the refolded/reactivated protein  
10 compared to the protein prior to inactivation/denaturation: this is termed "reconditional".

Preferably, the active site motif or an active site motif moiety includes the conserved sequence:

15

PLL(V)I(V)IA(S)EDV(I)EGEAL

in which amino acid symbols in parenthesis are alternatives to the immediately preceding symbol reading left to right.

20

- In seventh aspect the invention provides monomeric polypeptide having chaperone activity and incapable of multimerisation characterised in that in the absence of ATP the polypeptide has a protein refolding activity of more than 50%, preferably 60%, even more preferably 75%, said refolding activity being determined by contacting  
25 the polypeptide with an inactivated protein of known specific activity prior to inactivation, and then determining the specific activity of the said protein after contact with the polypeptide. the % refolding activity being:

$$\frac{\text{specific activity of protein after contact with polypeptide}}{\text{specific activity of protein prior to inactivation}} \times \frac{100}{1}$$

30

Preferably, the chaperone activity is determined by the refolding of cyclophilin A. More preferably, 8M urea denatured cyclophilin A (100 $\mu$ M) is diluted into 100mM potassium phosphate buffer pH7.0, 10mM DTT to a final concentration of 1 $\mu$ M and then contacted with at least 1 $\mu$ M of said polypeptide at 25°C for at least 5 min, the  
5 resultant cyclophilin A activity being assayed by the method of Fischer G *et al* (1984) Biomed Biochim Acta 43: 1101-1111.

The polypeptide is preferably an hsp60 polypeptide, preferably a GroEL polypeptide.

10

In eighth aspect the invention provides a polypeptide as claimed in any preceding claim which comprises at least an amino acid sequence selected from GroEL residues:

15 (a) 191-329, 191-330, 191-331, 191-332, 191-333, 191-334, 191-335, 191-336, 191-337, 191-338, 191-339, 191-340, 191-341, 191-342, 191-343, 191-344, 191-345, 191-346, 191-347, 191-348, 191-349, 191-350, 191-351, 191-352, 191-353, 191-354, 191-355, 191-356, 191-357, 191-358, 191-359, 191-360, 191-361, 191-362, 191-363, 191-364, 191-365, 191-366, 191-367, 191-368,  
20 191-369, 191-370, 191-371, 191-372, 191-373, 191-374, 191-375 or 191-376, or

(b) 192-329, 192-330, 192-331, 192-332, 192-333, 192-334, 192-335, 192-336, 192-337, 192-338, 192-339, 192-340, 192-341, 192-342, 192-343, 192-344,  
25 192-345, 192-346, 192-347, 192-348, 192-349, 192-350, 192-351, 192-352, 192-353, 192-354, 192-355, 192-356, 192-357, 192-358, 192-359, 192-360, 192-361, 192-362, 192-363, 192-364, 192-365, 192-366, 192-367, 192-368, 192-369, 192-370, 192-371, 192-372, 192-373, 192-374, 192-375 or 192-376, or

30

- (c) 193-329, 193-330, 193-331, 193-332, 193-333, 193-334, 193-335, 193-336, 193-337, 193-338, 193-339, 193-340, 193-341, 193-342, 193-343, 193-344, 193-345, 193-346, 193-347, 193-348, 193-349, 193-350, 193-351, 193-352, 193-353, 193-354, 193-355, 193-356, 193-357, 193-358, 193-359, 193-360, 193-361, 193-362, 193-363, 193-364, 193-365, 193-366, 193-367, 193-368, 193-369, 193-370, 193-371, 193-372, 193-373, 193-374, 193-375 or 193-376, or
- (d) 230-271, 229-271, 229-272, 228-272, 228-273, ...*et seq.* 194-328, 194-329, or

the equivalent residues of substantially homologous chaperonins, or a modified, mutated or variant sequence thereof.

- 15 A preferred polypeptide has the amino acid sequence 191-345 or 191-376, more preferably 193-335 or 191-337 of GroEL, or the equivalent residues of substantially homologous chaperonins, or a modified, mutated or variant sequence thereof.

The polypeptide preferably has a molecular weight of less than 34kDa.

- 20 "Modifications" include chemically modified polypeptides for example. "Variants" include, for example, naturally occurring variants of the kind to be found amongst a population of hsp60 chaperonin harbouring organisms/cells as well as naturally occurring polymorphisms or mutations. "Mutations" may also be introduced
- 25 artificially by processes of mutagenesis well known to a person skilled in the art.

- In being "substantially homologous" peptides may have at least 50% amino acid sequence homology with the specified GroEL amino acid sequences, preferably at least 60% homology and more preferably 75% homology. Homology may of
- 30 course also reside in the nucleotide sequences for the polypeptide which may be at

least 50%, preferably at least 60% homologous and more preferably 75% homologous with the nucleotide sequence encoding the specified GroEL amino acid residues.

- 5 The hsp60 class of chaperonin proteins are generally homologous in structure and so there are therefore conserved or substantially homologous amino acid sequences between the members of the class. GroEL is just an example of an hsp60 chaperonin protein; other suitable proteins having an homologous apical domain may be followed.

10

The list was compiled from the OWL database release 28.1. The sequences listed below show clear homology to apical domain (residues 191-375) in PDB structure pdb1grl.ent.

- 15 OWL is a non redundant database merging SWISS-PROT, PIR (1-3), GenBank (translation) and NRL-3D.

- 190-374 CH60\_ECOLI 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (AMS). - ESCHERICHIA 190-374 CH60\_SALTI 60 KD  
 20 CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - SALMONELLA TYPHI. 191-375 S56371 GroEL protein - Escherichia coli  
 190-374 CH60\_LEPIN 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (HEAT SHOCK 58 KD PRO 191-375 S47530 groEL  
 protein - Porphyromonas gingivalis 190-374 LPNHTPBG  
 25 LPNHTPBG NID:g149691 - Legionella pneumophila (strain SVir) (library: 189-373 CH60\_ACTAC 60 KD CHAPERONIN  
 (PROTEIN CPN60) (GROEL PROTEIN). - ACTINOBACILLUS ACT 191-  
 375 JC4519 heat-shock protein GroEL - Pasteurella  
 multocida

- 191-375 CH60\_BRUAB 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - BRUCELLA ABORTUS. 191-375 CH60\_HAEIN 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - HAEMOPHILUS INFLUE 190-373 CH60\_CAUCR 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - CAULOBACTER CRESCE 190-374 CH60\_AMOPS 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - AMOEBA PROTEUS SYM 191-375 CH60\_HAEDU 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - HAEMOPHILUS DUCREY 191-375 CH61\_RHIME 60 KD CHAPERONIN A (PROTEIN CPN60 A) (GROEL PROTEIN A). - RHIZOBIUM ME 190-374 CH60\_LEGMI 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (58 KD COMMON ANTIGEN 191-375 CH60\_YEREN 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (HEAT SHOCK PROTEIN 6) 190-374 CH 63\_BRAJA 60 KD CHAPERONIN 3 (PROTEIN CPN60 3) (GROEL PROTEIN 3). - BRADYRHIZOBI 191-375 CH60\_PORGI 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - PORPHYROMONAS GING 191-375 S52901 heat shock protein 60K - Yersinia enterocolitica 191-375 S26423 heat shock protein 60 - Yersinia enterocolitica 191-375 RSU373691 RSU37369 NID: g1208541 - Rhodobacter sphaeroides strain=HR. 190-374 CH62\_BRAJA 60 KD CHAPERONIN 2 (PROTEIN CPN60 2) (GROEL PROTEIN 2). - BRADYRHIZOBI 191-375 CH60\_ACYPS 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (SYMBIONIN). - ACYRTH 191-375 CH63\_RHIME 60 KD CHAPERONIN C (PROTEIN CPN60 C) (GROEL PROTEIN C). - RHIZOBIUM ME 191-375 YEPHSPCRP1 YEPHSPCRP NID: g466575 - Yersinia enterocolitica DNA. 191-375 CH60\_BORPE 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - BORDETELLA PERTUSS 189-373 BRUGRO1 BRUGRO

- NID: g144106 - *Brucella abortus* (library: lambda-2001) DNA.
- 191-375 CH60\_PSEAE 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - *PSEUDOMONAS AERUGI* 190-374 CH60\_BARBA 60 KD
- 5 CHAPERONIN (PROTEIN CPN60) (IMMUNOREACTIVE PROTEIN BB65) (IMMUNO 191-375 BAOBB63A BAOBB63A NID: g143845 - *Bartonella bacilliformis* (library: ATCC 35685) 189-373 CH60\_BACST 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - *BACILLUS STEAROTHE* 188-372
- 10 190-373 CH60\_BORBU 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - *BORRELIA BURGENDORFE* 224-408 S26583 chaperonin hsp60 - maize 190-373 A49209 heat shock protein HSP60 - Lyme disease spirochete 224-408 MZECPN60B MZECPN60B NID: g309558 - *Zea mays* (strain B73) (library: Dash11 of P.S
- 15 189-373 CH60\_THEP3 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (HEAT SHOCK 61 KD PRO 188-372 CH60\_STAEP 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (HEAT SHOCK PROTEIN 6 189-373 CH60\_LACLA 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - *LACTOCOCCUS LACTIS* 188-374
- 20 CH61\_STRAL 60 KD CHAPERONIN 1 (PROTEIN CPN60 1) (GROEL PROTEIN 1) (HSP58). - *STRE* 191-375 CH60\_CHLPN 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - *CHLAMYDIA PNEUMONI* 224-408 MZECPN60A MZECPN60A NID: g309556 - *Zea mays* (strain B73) (library: Dach 11 of P. 190-373 HECHSPAB1
- 25 HECHSPAB NID: g712829 - *Helicobacter pylori* (individual\_isolate 85P) D 221-405 CH60\_ARATH MITOCHONDRIAL CHAPERONIN HSP60 PRECURSOR. - *ARABIDOPSIS THALIANA* (MOUS 224-408 CH60\_MAIZE MITOCHONDRIAL CHAPERONIN HSP60 PRECURSOR. - *ZEA MAYS* (MAIZE). 190-374
- 30 CH60\_CHLTR 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL

- PROTEIN) (57 KD CHLAMYDIAL HYP 189-373 CH60\_STAAU 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (HEAT SHOCK PROTEIN 6 189-373 CH60\_CLOPE 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - CLOSTRIDIUM PERFRI 212-397
- 5 HS60\_YEAST HEAT SHOCK PROTEIN 60 PRECURSOR (STIMULATOR FACTOR 1 66 KD COMPONENT) 217-403 CH60\_PYRSA 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - PYRENOMONAS SALINA 191-377 CH60\_EHRCH 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - EHRlichia CHAFFEEN 191-375
- 10 CHTGROE1 CHTGROE NID: g144503 - C.trachomatis DNA. 188-372 CH60\_THETH 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - THERMUS AQUATICUS 189-373 TAU294831 TAU29483 NID: g1122940 - Thermus aquaticus. 190-378 CH60\_RICTS 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (MAJOR
- 15 ANTIGEN 58) (5 189-375 SYCCPNC SYCCPNC NID: g1001102 - Synechocystis sp. (strain PCC6803,) DNA. 190-373 CPU308211 CPU30821 NID: g1016083 - Cyanophora paradoxa. 189-373 CH61\_MYCLE 60 KD CHAPERONIN 1 (PROTEIN CPN60 1) (GROEL PROTEIN 1). - MYCOBACTERIU 239-423
- 20 PSU21139 PSU21139 NID: g806807 - pea. 191-377 CH60\_COWRU 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - COWDRIA RUMINANTIU 245-429 RUBB\_BRANA RUBISCO SUBUNIT BINDING-PROTEIN BETA SUBUNIT PRECURSOR (60 KD CHAPERON 144-328 SCCPN60 SCCPN60 NID: g1167857 - rye.
- 25 153-338 CH60\_EHRRI 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (55 KD MAJOR ANTIGEN) 245-429 RUBB\_ARATH RUBISCO SUBUNIT BINDING-PROTEIN BETA SUBUNIT PRECURSOR (60 KD CHAPERON 235-419 ATU49357 ATU49357 NID: g1223909 - thale cress strain=ecotype Wassilewskija. 195-379 RUB1\_BRANA
- 30 RUBISCO SUBUNIT BINDING-PROTEIN ALPHA SUBUNIT (60 KD

- CHAPERONIN ALPHA 189-374 CH62\_SYNY3 60 KD CHAPERONIN 2  
(PROTEIN CPN60 2) (GROEL HOMOLOG 2). - SYNECHOCYSTI 178-  
362 RUBA\_RICCO RUBISCO SUBUNIT BINDING-PROTEIN ALPHA  
SUBUNIT (60 KD CHAPERONIN ALPHA 190-375 CH60\_ODOSI 60 KD  
5 CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - ODONTELLA  
SINENSIS 236-420 PSU21105 PSU21105 NID: g1185389 - pea.  
224-409 CH60\_BRANA MITOCHONDRIAL CHAPERONIN CH60\_BACSU 60  
KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - BACILLUS  
SUBTILIS. 191-375 CH60\_AGRTU 60 KD CHAPERONIN (PROTEIN  
10 CPN60) (GROEL PROTEIN). - AGROBACTERIUM TUME 191-375  
b36917 heat shock protein GroEL - Agrobacterium  
tumefaciens  
191-375 PAU17072 PAU17072 NID: g576778 - Pseudomonas  
aeruginosa. 191-375 CH60\_RHILV 60 KD CHAPERONIN (PROTEIN  
15 CPN60) (GROEL PROTEIN). - RHIZOBIUM LEGUMINO 187-373  
CH61\_STRCO 60 KD CHAPERONIN 1 (PROTEIN CPN60 1) (GROEL  
PROTEIN 1) (HSP58). - STRE 191-375 CH60\_COXBU 60 KD  
CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (HEAT SHOCK  
PROTEIN B 191-375 CH62\_RHIME 60 KD CHAPERONIN B (PROTEIN  
20 CPN60 B) (GROEL PROTEIN B). - RHIZOBIUM ME 191-375  
PSEGROESL1 PSEGROESL NID: g151241 - Pseudomonas  
aeruginosa (library: ATCC 27853) 189-372 CH61\_SYNY3 60 KD  
CHAPERONIN 1 (PROTEIN CPN60 1) (GROEL HOMOLOG 1). -  
SYNECHOCYSTI 189-373 CH60\_CLOTM 60 KD CHAPERONIN (PROTEIN  
25 CPN60) (GROEL PROTEIN) (HSP-60). - CLOSTRIDI 191-373  
CH60\_PSEPU 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL  
PROTEIN). - PSEUDOMONAS PUTIDA 190-373 CH60\_SYNP7 60 KD  
CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - SYNECHOCOCCUS  
SP. 190-374 CH60\_GALSU 60 KD CHAPERONIN (PROTEIN  
30 CPN60) (GROEL PROTEIN). - GALDIERIA SULPHURA 190-374



- CH60\_ZYMMO 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - ZYMOMONAS MOBILIS. 191-375 JC2564 heat shock protein groEL - Zymomonas mobilis
- 191-375 CH60\_CHRVI 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - CHROMATIUM VINOSUM 189-373 CH60\_MYCTU 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (65 KD ANTIGEN) (HEAT 191-375 CH60\_NEIME 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (63 KD STRESS PROTEIN 189-373 CH60\_TREPA 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (TPN60) (TP4 ANTIGEN) 190-374 CH60\_HELPY 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (HEAT SHOCK PROTEIN 6 191-375 CH60\_NEIGO 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (63 KD STRESS PROTEIN 222-406 CH61\_CUCMA MITOCHONDRIAL CHAPERONIN HSP60-1 PRECURSOR. -
- 15 CUCURBITA MAXIMA (PUMPKI 189-373 CH60\_MYCPA 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (65 KD ANTIGEN) (HEAT 230-414 MPU15989 MPU15989 NID:g559802 - Mycobacterium paratuberculosis. 224-408 S26582 chaperonin hsp60 - maize 191-375 S40247 heat-shock protein - Neisseria gonorrhoeae 189-373 CH60\_CLOAB 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN). - CLOSTRIDIUM ACETOB 191-375 CH60\_NEIFL 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (63 KD STRESS PROTEIN 190-373 CH60\_LEGPN 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL PROTEIN) (58 KD COMMON ANTIGEN 222-406 CH62\_CUCMA MITOCHONDRIAL CHAPERONIN HSP60-2 PRECURSOR. - CUCURBITA MAXIMA (PUMPKI 191-375 CHTGROESL1 CHTGROESL NID: g402332 - Chlamydia trachomatis DNA. 64-248 S40172 S40172 NID: g251679 - Chlamydia psittaci pigeon strain P-1041. 189-
- 30 373 SYOGROEL2 SYOGROEL2 NID:g562270 - Synechococcus

- vulcanus DNA. 191-375 CH60\_CHLPS 60 KD CHAPERONIN  
(PROTEIN CPN60) (GROEL PROTEIN) (57 KD CHLAMYDIAL HYP 188-  
372 CH62\_STRAL 60 KD CHAPERONIN 2 (PROTEIN CPN60 2) (GROEL  
PROTEIN 2) (HSP56). - STRE 189-373 CH62\_MYCLE 60 KD
- 5 CHAPERONIN 2 (PROTEIN CPN60 2) (GROEL PROTEIN 2) (65 KD  
ANTIGEN) 236-420 MSGANTM MSGANTM NID: g149923 - M.leprae  
DNA, clone Y3178.  
CPN60 PRECURSOR. - BRASSICA NAPUS (RAPE). 105-289 PMSARG2  
PMSARG2 NID: g607157 - Prochlorococcus marinus.
- 10 234-417 RUB2\_BRANA RUBISCO SUBUNIT BINDING-PROTEIN ALPHA  
SUBUNIT PRECURSOR (60 KD CHAPERO 75-259 CRECPN1A CRECPN1A  
NID: g603910 - Chlamydomonas reinhardtii cDNA to mRNA.  
215-400 P60\_CRIGR MITOCHONIDRIAL MATRIX PROTEIN P1  
PRECURSOR (P60 LYMPHOCYTE PROTEIN) (CH224-408 CRECPN1B
- 15 CRECPN1B NID: g603912 - Chlamydomonas reinhardtii cDNA to  
mRNA. 191-375 RUBA\_WHEAT RUBISCO SUBUNIT BINDING-PROTEIN  
ALPHA SUBUNIT PRECURSOR (60 KD CHAPERO 189-373 B47292  
heat shock protein groEL - Mycobacterium tuberculosis  
206-391 CELHSP60CP CELHSP60CP NID: g533166 -
- 20 Caenorhabditis elegans (strain CB1392) cDNA 215-400  
P60\_HUMAN MITOCHONDRIAL MATRIX PROTEIN P1 PRECURSOR (P60  
LYMPHOCYTE PROTEIN) (CH 215-400 P60\_MOUSE MITOCHONDRIAL  
MATRIX PROTEIN P1 PRECURSOR (P60 LYMPHOCYTE PROTEIN) (CH  
215-400 P60\_RAT MITOCHONDRIAL MATRIX PROTEIN P1 PRECURSOR
- 25 (P60 LYMPHOCYTE PROTEIN) (CH 215-400 A41931 chaperonin  
hsp60 - mouse  
197-382 MMHSP60A MMHSP60A NID: g51451 - house mouse. 218-  
402 CH63\_HELVI 63 KD CHAPERONIN PRECURSOR (P63). -  
HELIOTHIS VIRESCENS (NOCTUID MOTH) 205-390 EGHSP60GN
- 30 EGHSP60GN NID: g1217625 - Euglena gracilis. 222-407

- HS60\_SCHPO PROBABLE HEAT SHOCK PROTEIN 60 PRECURSOR. -  
 SCHIZOSACCHAROMYCES POMBE 198-385 S61295 heat shock  
 protein 60 - Trypanosoma cruzi  
 198-385 TRBMTHSP TRBMTHSP NID: g903883 - Mitochondrion
- 5 Trypanosoma brucei (strain EATRO 8-69 ECOGROELA ECOGROELA  
 NID: g146268 - E.coli DNA, clone E. 142-325 ENHCPN60P  
 ENHCPN60P NID: g675513 - Entamoeba histolytica (strain  
 HM-1:IMSS) DNA. 257-433 CH60\_PLAFG MITOCHONDRIAL  
 CHAPERONIN CPN60 PRECURSOR. - PLASMODIUM FALCIPARUM (ISO
- 10 1-90 CRECPN1C CRECPN1C NID: g603914 - Chlamydomonas  
 reinhardtii cDNA to mRNA.  
 5-65 ATTS0779 ATTS0779 NID: g17503 - thale cress.  
 189-373 CH60\_MYCGE 60 KD CHAPERONIN (PROTEIN CPN60) (GROEL  
 PROTEIN). - MYCOPLASMA GENITAL 228-411 HTOHSP60X
- 15 HTOHSP60X NID: g553068 - Histoplasma capsulatum (strain  
 G217B) DNA. 190-297 CH60\_SYNP6 60 KD CHAPERONIN (PROTEIN  
 CPN60) (GROEL PROTEIN) (FRAGMENT). - SYNECHO 169-245  
 RUBA\_ARATH RUBISCO SUBUNIT BINDING-PROTEIN ALPHA SUBUNIT  
 (60 KD CHAPERONIN ALPHA

20

- The polypeptide may further comprise a polyamino acid sequence, preferably an N-terminal polyamino acid sequence although a C-terminal sequence may be present
- 25 instead or in addition to an N-terminal sequence. The polyamino acid sequence may be selected from the same or different amino acid residues. When the same amino acid residue is repeated a particularly preferred polyamino acid sequence is a polyhistidine sequence.

Whether composed of the same or different amino acid residues, the further polyamino acid sequence may comprise any number of amino acid residues so long as chaperonin activity is provided. The other amino acid residues which may be included in the further polyamino acid sequence could be selected from any of the  
5 twenty or so essential amino acids common to biological systems or of any amino acid variant or derivative. When the polyamino acid sequence is other than a homopolymer then it may comprise a repeated sequence of two or more amino acid residues. The amino acid sequence may encode a portion of another known protein or polypeptide or it may even be random.

10

The further polyamino acid sequence preferably also includes a cleavage site cleavable by a cleavage agent. A preferred cleavage agent is thrombin although any other suitable agent will suffice. The sequence of amino acid residues is of course selected to permit cleavage by the desired cleavage agent.

15

The further polyamino acid sequence preferably comprises 17 to 39 amino acids although more than 39 and less than 17 amino acids may also be employed.

20

The polypeptide may be attached to a support, preferably in immobilised form, optionally immobilised to a chromatographic matrix, more preferably an agarose resin. When an agarose resin is used it is preferably a nickel-nitrilo-tri-acetic acid (NTA)-ligated agarose resin. This has affinity for a polypeptide having a polyhistidine tail.

25

The polypeptide of the invention may be obtained by recombinant means. Alternatively, the polypeptide may be produced by a routine chemical synthesis using standard polypeptide synthesis procedures known in the art. If produced by recombinant means the polypeptide may be fused to a heterologous protein or polypeptide.

30

The inventors have found that the fragments with polyhistidine tails of 17 amino acid residues sht-GroEL 193-335, sht-GroEL191-345 and sht-GroEL191-376 as well as GroEL191-345 are potent facilitators of the folding of inactive forms of cyclophilin A and rhodanese, and catalyse the unfolding of barnase. Maximal  
5 refolding yield was obtained at stoichiometric concentrations of cyclophilin A and apical domain, indicating the formation of a 1:1 complex between chaperone fragment and substrate protein during refolding. The fragments sht-GroEL 193-335, sht-GroEL191-345 and sht-GroEL191-376 when attached to nickel-nitrilotriacetic acid (NTA)-ligated agarose resin or NHS agarose resin are also active in  
10 enhancing the folding yield of destabilised mutants of indole glycerol-phosphate synthase and of the protein cyclophilin A. This proves the monomer has refolding activity and also demonstrates that the immobilised protein is functionally useful.

Fragments of GroEL may be produced either from recombinant DNA methods or  
15 from protein chemistry or any means of chemical synthesis, or the equivalent fragments of homologous hsp60 (cpn60) proteins or any natural variants or any variants produced by mutagenesis, or of larger fragments containing the sequences 191-376, 191-345 or 193-335 of GroEL from *E. coli*.

20 In tenth aspect the invention provides a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide of the invention, or a nucleotide sequence hybridisable thereto and optionally encoding a polypeptide having chaperone activity.

25 Preferred characteristics of the nucleotide sequence correspond to the preferred features of the polypeptides of the aspects of the invention as hereinbefore defined.

This aspect of the invention therefore includes a recombinant DNA molecule for use in cloning and/or expressing a DNA sequence, said recombinant DNA molecule  
30 comprising:

- (a) a nucleotide sequence encoding amino acid residues 191-376 of GroEL,
- (b) a nucleotide sequence encoding amino acid residues 191-345 of GroEL,
- 5 (c) a nucleotide sequence encoding amino acid residues 193-337 of GroEL,
- (d) a nucleotide sequence encoding amino acid residues 193-335 of groEL,
- 10 (e) a nucleotide sequence encoding amino acid residues of GroEL selected from amino acid residues:
- (i) 191-329, 191-330, 191-331, 191-332, 191-333, 191-334, 191-335, 191-336, 191-337, 191-338, 191-339, 191-340, 191-341, 191-342, 191-343, 191-344, 15 191-345, 191-346, 191-347, 191-348, 191-349, 191-350, 191-351, 191-352, 191-353, 191-354, 191-355, 191-356, 191-357, 191-358, 191-359, 191-360, 191-361, 191-362, 191-363, 191-364, 191-365, 191-366, 191-367, 191-368, 191-369, 191-370, 191-371, 191-372, 191-373, 191-374, 191-375 or 191-376, or
- 20 (ii) 192-329, 192-330, 192-331, 192-332, 192-333, 192-334, 192-335, 192-336, 192-337, 192-338, 192-339, 192-340, 192-341, 192-342, 192-343, 192-344, 192-345, 192-346, 192-347, 192-348, 192-349, 192-350, 192-351, 192-352, 192-353, 192-354, 192-355, 192-356, 192-357, 192-358, 192-359, 192-360, 25 192-361, 192-362, 192-363, 192-364, 192-365, 192-366, 192-367, 192-368, 192-369, 192-370, 192-371, 192-372, 192-373, 192-374, 192-375 or 192-376. or
- (iii) 193-329, 193-330, 193-331, 193-332, 193-333, 193-334, 193-335, 193-336, 30 193-337, 193-338, 193-339, 193-340, 193-341, 193-342, 193-343, 193-344,

193-345, 193-346, 193-347, 193-348, 193-349, 193-350, 193-351, 193-352,  
193-353, 193-354, 193-355, 193-356, 193-357, 193-358, 193-359, 193-360,  
193-361, 193-362, 193-363, 193-364, 193-365, 193-366, 193-367, 193-368,  
193-369, 193-370, 193-371, 193-372, 193-373, 193-374, 193-375 or 193-  
5 376.

(f) 230-271, 229-271, 229-272, 228-272, 228-273, ...*et seq*... 194-328, 194-  
329, or

10 (g) a nucleotide sequence hybridisable to any of (a), (b), (c), (d), (e) or (f) above  
and encoding a monomeric polypeptide having chaperone activity, or

(h) degenerate nucleotide sequences corresponding to (a), (b), (c), (d), (e), (f) or  
(g) above.

15

Further features of the nucleic acid sequence may be as described hereinbefore in  
relation to the polypeptides of the invention; the nucleic acid sequence having the  
appropriate nucleotide sequence which on expression provides a polypeptide which  
possesses these features.

20

In eleventh aspect the invention provides a vector comprising a nucleic acid as  
hereinbefore defined.

25

In twelfth aspect the invention provides a host cell transformed with a vector or a  
nucleic acid molecule as hereinbefore defined.

30

In thirteenth aspect there is provided a method of making a polypeptide of the  
invention comprising transforming a host cell with a nucleic acid encoding said  
polypeptide, culturing the transformed cell for and expressing said polypeptide.  
Expression may be direct or as a fusion product. When the polypeptide product is

expressed as a fusion then the method preferably includes a step wherein the expressed polypeptide product is subject to cleavage. The polypeptide may be recovered from the transformed cells expressing it.

- 5 In fourteenth aspect the invention provides a pharmaceutical formulation comprising a polypeptide of the invention, optionally together with a diluent, carrier or excipient.

- The active ingredients of a pharmaceutical composition comprising the polypeptide  
10 are contemplated to exhibit excellent therapeutic activity, for example, in the alleviation of Alzheimer's disease when administered in amount which depends on the particular case. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of  
15 the therapeutic situation.

- The active compound may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, intranasal, intradermal or suppository routes or implanting (eg using slow release molecules).  
20 Depending on the route of administration, the active ingredient may be required to be coated in a material to protect said ingredients from the action of enzymes, acids and other natural conditions which may inactivate said ingredient.

- In order to administer the polypeptide by other than parenteral administration, it  
25 will be coated by, or administered with, a material to prevent its inactivation. For example, the polypeptide may be administered in an adjuvant, co-administered with enzyme inhibitors or in liposomes. Adjuvant is used in its broadest sense and includes any immune stimulating compound such as interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as



polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether. Enzyme inhibitors include pancreatic trypsin.

5 Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes.

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these  
10 preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases  
15 the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid  
20 polyethethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

25 The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the

compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

- 5 Sterile injectable solutions are prepared by incorporating the active compound in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilisation. Generally, dispersions are prepared by incorporating the sterilised active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the
- 10 preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and the freeze-drying technique which yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.
- 15 When the polypeptide is suitably protected as described above, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsules, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet. For oral therapeutic administration, the active compound may be incorporated with
- 20 excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained.
- 25 The tablets, troches, pills, capsules and the like may also contain the following: a binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such
- 30 as peppermint, oil of wintergreen, or cherry flavouring. When the dosage unit form

is a capsule, it may contain, in addition to materials of the above type, a liquid carrier.

Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound may be incorporated into sustained-release preparations and formulations.

As used herein "pharmaceutically acceptable carrier and/or diluent" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such as active

material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired.

5 The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

10 In ninth aspect there is provided polypeptide of the invention as hereinbefore defined for use in the treatment of disease. Consequently there is provided the use of a polypeptide of the invention for the manufacture of a medicament for the treatment of disease associated with aberrant protein/polypeptide structure. The aberrant nature of the protein/polypeptide may be due to misfolding or unfolding which in turn may be due to an anomalous eg mutated amino acid sequence. The  
15 protein/polypeptide may be destabilised or deposited as plaques eg as in Alzheimer's disease. The disease might be caused by a prion. A polypeptide-based medicament of the invention would act to renature or resolubilise aberrant, defective or deposited proteins.

20 In fifteenth aspect there is provided a nucleic acid molecule in accordance with other aspects of the invention for use in the treatment of disease. Consequently, there is provided the use of a nucleic acid molecule of the invention for the manufacture of a medicament for the treatment of disease associated with protein/polypeptide structure. Genetic therapy *in vivo* is therefore provided for by  
25 way of introduction and expression of DNA encoding the monomeric polypeptide in cells/tissues of an individual to provide chaperonin activity in those cells/tissues.

In sixteenth aspect there is provided the use of a polypeptide of the invention for altering the structure of a molecule, particularly a protein or polypeptide and the  
30 alteration in structure may be by folding, unfolding or resolubilising. Preferably,

the stoichiometry between the monomeric polypeptide of the invention and the molecule being altered is about 1:1, preferably 1:1.

In seventeenth aspect there is provided a method of reconditioning a molecule  
5 preferably a protein comprising contacting said protein with a polypeptide of the invention. Preferably the protein is subjected to inactivation or denaturation prior to contacting with said polypeptide. The polypeptide may be immobilised to a solid phase, preferably a chromatographic matrix, and the contacting of protein and polypeptide is carried out by applying the protein to the top of a bed of the matrix  
10 packed in a column and then eluting the polypeptide through the column.

This aspect of the invention therefore provides for the use of a polypeptide as hereinbefore defined for altering the structure of a molecule, preferably a protein or polypeptide and the alteration in structure is by folding, unfolding or refolding.  
15 The stoichiometry between the polypeptide and the molecule being altered may be about 1:1.

In nineteenth aspect the invention provides for the use of a polypeptide of the invention the purification or increase in yield, specific activity or quality of  
20 biological molecules, preferably said polypeptide being attached to a support.

In twentieth aspect the invention provides a kit for reconditioning or refolding a molecule, preferably a protein, comprising a polypeptide as hereinbefore defined immobilised to a solid phase and a container for holding said solid phase  
25 polypeptide.

In twenty-first aspect the invention provides for the use of a polypeptide as hereinbefore defined in the production of a protein or polypeptide by recombinant means, wherein the said polypeptide is co-expressed with the protein or polypeptide  
30 thereby to improve the yield or quality of the protein or polypeptide.

In twenty-second aspect the invention provides an antibody reactive against a polypeptide of the invention. The invention also provides for the use of the antibody in the treatment of disease. Also, the invention provides for the use of the antibody in the manufacture of a medicament for the treatment of disease associated with protein/polypeptide structure.

In twenty-third aspect the invention provides various methods of treating disease, namely a method of treating disease in which an effective amount of a polypeptide of the invention is administered. There is also a method of treating disease which comprises administering an effective amount of an inhibitor of the chaperone activity of a polypeptide of the invention. Preferably, said inhibitor is an antibody. Also, there is a method of treating disease by gene therapy which utilises a construct encoding a polypeptide of the invention or an antagonist thereof.

The monomeric apical domain of GroEL or its fragments catalyse amide proton exchange of barnase, and facilitate refolding of rhodanese and cyclophilin A in the absence of cofactors. Thus, GroEL has an intrinsic chaperone activity, which is not restricted to its oligomeric state or central cavity. The C-terminal  $\alpha$ -helices of the apical domain are not directly involved in polypeptide binding, and form a separate folding unit. At physiological temperature, about 50% of the C-terminus is unfolded, allowing high mobility movements of the polypeptide binding "domain core". Flexibility within the apical domain may be crucial for cooperative binding of a wide range of proteins with different native structure, and for the conformation change of GroEL on binding to the co-chaperonin GroES. The crystal structure of the "domain core" of the apical domain has an overall *B*-factor 60% lower than that of the same region in intact GroEL. The overall fold of the fragment is similar to the corresponding region of intact GroEL, but the amount of secondary structure is considerably larger. There are three  $3_{10}$ -helices in the structure of the fragment, which are involved in binding of polypeptide and/or GroES.

The inventors have expressed the apical domain of GroEL as a stable monomeric protein and with high yield in *E. coli*, allowing its activity, folding, and structure to be studied separately from those of the equatorial and intermediate domains. The isolated, apical domain is functional in polypeptide binding (Figure 2), and it facilitates protein folding even when truncated to remove its C-terminal  $\alpha$ -helices, H11 and H12 (Figure 3). The apical domain slows down spontaneous refolding of rhodanese and cyclophilin A by >15-fold and >150-fold, respectively (Figures 3c-e). The inventors have shown increased refolding yield of rhodanese and cyclophilin A in the presence of apical domain (Figure 3), which is too small to reassociate and to form a cavity, clearly demonstrates the presence of an intrinsic chaperone activity in GroEL, which is independent of a central cavity. This is in agreement with NMR experiments on barnase in the presence of apical domain (Figure 2). The presence of micromolar concentrations of GroEL considerably broadens the resonance lines of barnase owing to a fast association and idssociation of native barnase from the slowly rotational tumbling GroEL (Zahn *et al* (1996) Science 271: 642-645). If the apical domain and barnase would form a complex of large molecular size, i.e. containing seven or 14 molecules of apical domain, one would expect a considerable degree of line broadening in the NMR spectra of barnase in the presence of apical domain. However, this is not what was observed, even not at eight-fold higher monomer concentration of apical domain than of GroEL (Zahn *et al* (1996) Science 271: 642-645). The complex between apical domain and barnase, therefore, rather seems to be of low molecular weight and probably has a 1:1 stoichiometry, which would be consistent with the binding stoichiometry of the apical domain and cyclophilin A (Figure 3e).

The intrinsic chaperone activity of monomeric apical domain facilitates refolding of rhodanese even though physiological conditions, GroES, and ATP are required in the presence of intact GroEL. Although the inventors do not wish to be bound by any particular hypothesis the role of GroES appears to be to weaken the affinity of

GroEL for substrates, and to prevent premature dissociation of aggregation prone states: there has to be a balance between tight binding for the annealing activity and weaker binding to allow folding (Corrales F J and Fersht A R (1996) Proc Natl Acad Sci USA 93: 4509-4512). The weaker binding of rhodanese to the fragment  
5 must be adequate for chaperoning activity and weak enough for folding. This is in agreement with the observed similar rate constant for refolding of rhodanese in the presence of apical domain and in the presence of GroEL, GroES, and ATP (Figures 3b-d), indicating a similar binding affinity. The complex structure of GroEL, and the modulation of its substrate affinity by GroES and nucleotides, must be to allow  
10 the efficient folding of a wide range of proteins, with a wide variation of affinities for GroEL. The mechanism, in general, may involve several components: GroES binds to the *cis*-end of a GroEL-substrate complex and displaces the bound polypeptide into the cavity; the dissociated and encapsulated polypeptide chain then refolds in an enlarged folding cavity to a native-like state, until it is released upon  
15 the dissociation of GroES; and, the cycle of binding and dissociation of unfolded protein, GroES, ATP, and GroEL may be repeated several times, before the substrate protein can adopt a native conformation (Hunt *et al* (1996) Nature 379: 37-45; Weissman *et al* (1996) Cell 84: 481-490; Weissman J S *et al* (1995) Cell 83: 577-587; Mayhew M *et al* (1996) Nature 379: 420-426; Corrales F J and Fersht A  
20 R (1996) Proc Natl Acad Sci USA 93: 4509-4512). Polypeptides with intermediate affinity for GroEL, such as cyclophilin A (Figure 3e), bind transiently to GroEL and folding is facilitated without the need for co-factors.

Helices H11 and H12 are much less stable than the "domain core" of the apical  
25 domain, and form a separate folding unit (Figure 4). At physiological temperature, about 50% of the secondary structure of the low-melting helices is unfolded and thus becomes flexible. But, *in vivo*, the C-terminus is not degraded, implying that the primary structure is not accessible to degradation by *E. coli* proteases. The low stability of the C-terminal helices allows movements and larger fluctuations of the  
30 "domain cores" within the GroEL-ring. This flexible arrangement of the apical



- domain may be implicated in the cooperative binding of unfolded polypeptide to the seven subunits of GroEL in each ring. It is understandable that flexibility is required for binding to a large variety of proteins with different amino acid sequence, and secondary and tertiary structure. The observed low stability of the C-terminus may also contribute to the large conformational change in GroEL on binding of GroES, which has been suggested to occur through rigid body movement through the  $\beta$ -sheet hinge region of the intermediate domain (Braig K *et al* (1994) Nature 371: 578-586).
- 10 The production and crystallisation of monomeric fragments of the apical domain by the inventors has allowed a determination of the three-dimensional structure of the polypeptide binding part of GroEL with *B*-factors much lower than those of the equivalent regions in GroEL (Figure 5) (Braig K *et al* (1994) Nature 371: 578-586; Braig *et al* (1995) Nature Struct Biol 2: 1083-1094; Boisvert *et al* (1996) Nature
- 15 Structure Biology 3: 170-177), perhaps because of favourable packing interactions within the crystal, or the absence of the flexible C-termini in the GroEL fragment, or a combination of both. Particularly interesting is the existence of  $3_{10}$ -helices within the apical domain. These tightly packed helices are much less common than  $\alpha$ -helices, and the majority are very short, with 96% being less than four residues.
- 20 They are usually found at the end of  $\alpha$ -helices, where the final turn may have this conformation, or between two  $\beta$ -strands. All the  $3_{10}$ -helices of the apical domain are localised within regions, which have been shown to be involved in polypeptide and/or GroES binding (Fenton *et al* (1994) Nature 371: 614-619). The third  $3_{10}$ -helix has an unusual length of 10 residues. A  $3_{10}$ -helix with a similar length (9
- 25 residues) has been found in aconitase (Robbins *et al* (1989) Proteins: Structure, Function and Genetics 5: 289-312), an enzyme of the Calvin cycle. Interestingly, the apical domain and aconitase also share a similar fold: a central  $\beta$ -sheet linked by  $\alpha$ -helices, suggesting that they may use a common mechanism for substrate binding.

Preferred embodiments of the invention will now be described in detail by way of the following examples and with reference to the accompanying drawings in which:

Figure 1 shows the amino acid sequence of the apical region of GroEL from *E. coli*  
5 (SEQ ID NO:9) including further amino acid residues providing an N-terminal histidine-tail (ht)(SEQ ID NO:8).

Figure 2a is a bar chart showing the catalytic effect of GroEL 191-345 on the amide proton exchange of unfolded barnase. The ratio of the protection factors of the 39  
10 measurable amide protons of barnase in the absence (P) and presence (P+(G)) of the GroEL fragment is shown.

Figure 2b is a bar chart similar to that of Figure 2a but for the GroEL fragment 191-345 with a 17 residue N-terminal histidine tail (sht-GroEL 191-345).

15 Figure 2c is a bar chart similar to that of Figure 2a but for the GroEL fragment 191-376 with a 17 residue N-terminal histidine tail (sht-GroEL 191-376).

Figure 2d is a plot of observed exchange rate constants in the absence of chaperonin  
20 ( $k_{\text{ex}}^{\text{obs}}$ ) against those in the presence of chaperonin ( $k_{\text{ex}}^{\text{obs}}(+G)$ ) for GroEL fragment 191-345. Global, mixed and local amide protons are displayed by circles, triangles and squares respectively.

Figure 2e is a plot similar to that of Figure 2d but for sht-GroEL 191-345.

25 Figure 2f is a plot similar to that of Figure 2d but for sht-GroEL 191-376.

Figure 3a is a bar chart and table showing the relative enzymatic activity of rhodanese after refolding in the presence or absence of GroEL, GroES, ATP, sht-  
30 GroEL 191-345, sht-GroEL 191-376 or bovine serum albumin (BSA).

Figure 3b is a plot showing the refolding kinetics of rhodanese in the presence of GroEL, GroES and ATP.

- 5 Figure 3c is a plot showing the refolding kinetics of rhodanese in the presence of various concentrations of sht-GroEL 191-345.

Figure 3d is a plot showing the refolding kinetics of rhodanese in the presence of various concentrations of sht-GroEL 191-376.

10

Figure 3e is a plot comparing the refolding kinetics of specified concentrations of sht-GroEL 191-376, sht-GroEL 191-345 and GroEL.

- 15 Figure 4a is a trace of the thermal denaturation of sht-GroEL 191-376 (upper trace) and sht-GroEL 191-345 (lower trace) monitored by far ultra violet-circular dichroism at 222nm.

Figure 4b is a trace as in Figure 4a but thermal denaturation is monitored by differential scanning calorimetry.

20

Figure 5a shows a three dimensional representation of the structure of sht-GroEL 191-345.

- 25 Figure 5b shows a three dimensional representation of the backbone structure of sht-GroEL 191-345.

Figure 5c shows a three dimensional representation of electron densities of sht-GroEL 191-345 viewed along the helices H8 and H9.

Figure 6 is a trace showing the elution of denatured mutant IGPS from a column of immobilised GroEL 191-345 developed with refolding buffer.

Figure 7 shows the amino acid sequence of GroEL from *E. coli* numbered from the  
5 N-terminal methionine (SEQ ID NO:10).

Figure 8 shows the amino acid sequence of a portion of GroEL wherein the residues involved in binding peptides are highlighted (SEQ ID NO:11).

10 Figures 9a-c comprise partial amino acid sequences of known cpn 60 family members aligned with one another for comparison.

Example 1 - Cloning and expression of the apical domain of GroEL and various fragments thereof

15

The apical domain of GroEL (GroEL 191-376) and various C-terminally truncated fragments of the apical domain are cloned by polymerase chain reaction (PCR) into the polylinker site of a pRSET A vector (Invitrogen), coding for an N-terminal histidine-tail, which contains an engineered thrombin cleavage site. The histidine-tail is composed of 36 amino acids (Invitrogen) or 17 amino acids. For the PCR  
20 reaction, the plasmid pOF39 (Fayet O *et al* (1989) J Bacteriol 171: 1379-1385) is used as a template together with two primers flanking the respective GroEL fragment with *Bam*H1 and *Ceo*RI restriction enzyme sites. This permits the cloning of the PCR fragment into the polylinker of pRSET A. PCR is performed with *Pfu*  
25 (Stratagene) to reduce the risk of undesired random mutations. The reaction is performed in a volume of 25  $\mu$ l for 25 cycles with 400 nM or primer and 200  $\mu$ M of deoxynucleoside-5'-triphosphates. The annealing temperature is 65°C. The following primers are used for the PCT to generate DNA encoding the apical region of GroEL and a variety of fragments thereof: 5' flanking: 5'-CGG ATC CGA AGG  
30 TAT GCA GTT CGA CCG (SEQ ID NO:1); 3' flanking (s)ht-GroEL191-376, 5'-

CGA ATT CTT AAA CGC CGC CTG CCA GTT TCG (SEQ ID NO:2); 3'  
flanking (s)ht-GroEL191-345, 5'-CGA ATT CTT AAC GGC CCT GGA TTG  
CAG CTT C (SEQ ID NO:3); 3' flanking ht-GroEL191-337, 5'-CGA ATT CTT  
AAC CCA CGC CAT CGA TGA TAG TGG TG (SEQ ID NO:4); 3' flanking ht-  
5 GroEL191-328, 5'-CGA ATT CTT AGT CTT TGT TGA TCA CAA CAC GTT  
TAG CCT GAC (SEQ ID NO:5); 3' flanking ht-GroEL191-322, 5'-CGA ATT  
CTT AAC GTT TAG CCT GAC CCA GGT CTT CCA (SEQ ID NO: 6); 3'  
flanking ht-GroEL191-298, 5'-CGA ATT CTT AAC CGC CAG TCA GGG TTG  
CGA TAT C (SEQ ID NO: 7).

10

The following protocol is used for expression and purification of the fragments of  
GroEL in *E. coli* TG2 cells. With reference to Figure 1, the expression vector used  
codes for an N-terminal histidine-tail (ht) composed of 36 amino acids and  
containing a thrombin cleavage site (vertical arrow). Alternatively, a shorter  
15 version of this histidine-tail (sht) containing 17 amino acids is used. The N- and C-  
terminal ends of the generate fusion proteins, namely ht-GroEL 191-298, ht-GroEL  
191-322, ht-GroEL 191-328, ht-GroEL 191-337, ht-GroEL 191-345, ht-GroEL  
191-376, sht-GroEL 191-345, and sht-GroEL 191-376 are indicated by rectangular  
arrows.

20

Two litres of L-Broth medium plus ampicillin is inoculated 1:100 with an over-night  
culture of TG2 cells containing the respective plasmid. At an  $A_{600}$  of 0.3,  
expression is induced with isopropyl-1-thio- $\beta$ -D-galactopyranoside to 0.2 mM final  
concentration and M13/T7-phage at a multiplicity of infection of 10 phages per cell.  
25 The cells are harvested 8 h after induction, centrifuged, and resuspended in 200 ml  
buffer A (50 mM Tris-HCl pH 8.2, 300 mM NaCl).

After sonication and centrifugation, the soluble protein fraction is added to 20 ml  
nickel-NTA agarose resin and stirred for 10 min. The resin is washed with 200 ml  
30 buffer A, and the histidine-tail containing fusion protein is eluted with 50 ml buffer

A containing 200 mM imidazole. The eluted protein is precipitated with 80% ammonium sulfate, re-dissolved in 4 ml of buffer B (50 mM Tris-HCl pH 8.2, 150 mM sodium chloride), and is loaded on a HiLoad 26/60 Superdex 75 column (Pharmacia), which is equilibrated with buffer B. The fragment GroEL 191-345 is produced by thrombin-cleavage of ht-GroEL 191-376 before gel filtration. The cleaving reaction is carried out for several days in buffer B after addition of 250  $\mu$ l thrombin (Sigma, 1U/ $\mu$ l) to the protein solution eluted from the nickel-NTA column. The GroEL fragments are analyzed by quantitative amino acid analysis, N-terminal sequencing, and mass spectrometry.

10

In Figure 1, secondary structure is indicated by boxes and arrows for  $\alpha$ -helix (grey) or  $3_{10}$ -helix (white) and  $\beta$ -sheet structure, respectively. Assignment of secondary structure of residues 191 to 336 is done on the basis of the crystal structure of sht-GroEL 191-345 (Table 1 and Fig 5) using PROCHECK (Laskowski R A *et al* (1993) J Appl Cryst 26: 283-291) and the algorithm of Kabsch & Cander (Kabsch W and Sander C (1983) Biopolymers 22: 2577-2637). Numbering of  $\alpha$ -helices and secondary assignment of residues 337 to 376 according to Braig *et al* (Braig K *et al* (1995) Nature Struct Biol 2: 1083-1094).

20 Functional apical domain of GroEL and various functional fragments of the apical domain in *E. coli* are expressed allowing the study of chaperone activity, folding, and crystal structure of the polypeptide binding part of GroEL. In particular, the apical domain of GroEL (GroEL 191-376) and various fragments of the apical domain (Fig 1) are expressed in *E. coli* as fusion proteins containing an N-terminal histidine-tail, and this allowed for a straightforward purification using a nickel-nitrilo-tri-acetic acid (NTA)-ligated agarose resin. The histidine-tail of either 39 (ht) or 17 amino acids (sht) contains a sequence of six histidine residues and a thrombin cleavage site. The apical domain is expressed at >20 mg purified protein per litre of culture as are the smaller fragments lacking the C-terminal  $\alpha$ -helices, H11 and H12. Further truncation before residue 329 leads to considerable

25  
30

destabilisation of the apical domain. The fragment GroEL 191-345, containing no histidine-tail and without the two helices is generated by thrombin cleavage of purified ht-GroEL 191-376.

- 5 The apical region and monomeric fragments are found to have chaperone activity. The circular dichroism (CD) of sht-GroEL 191-376, sht-GroEL 191-345, and GroEL 191-345 in the far ultra violet (UV) region and in the near UV region indicated native-like secondary and tertiary structure, respectively. The apical domain and the fragment truncated at position 345 are monomeric at micromolar
- 10 concentrations, when determined by ultracentrifugation. However, the line widths in the nuclear magnetic resonance (NMR) spectra of GroEL 191-345 are larger than expected for a 17 kD protein but small than for a stable dimer (data not shown), indicating a fast intermolecular interaction between monomers on the NMR time scale (Wüthrich, K, NMR of Proteins and Nucleic Acids (Wiley, New York, 1986).
- 15 There seems to be a low affinity self-recognition of the apical domain.

Example 2 - Binding of GroEL 191-345, sht-GroEL 191-345, and sht-GroEL 191-376 to unfolded barnase

- 20 Exchange experiments are carried out at 33°C in 20 mM imidazole buffer as described in Zahn *et al* (1996) Science 271: 642-645. The concentration of barnase is 2.4 mM. The results are shown in Figures 2a, 2b and 2c; locally and globally exchanging amide protons, and amide protons exchanging by a mixture of both mechanisms (Perrett *et al* (1995) Biochemistry 34: 9288-9298 and Clarke *et al*
- 25 (1993) Proc Natl Acad Sci USA 90: 9837-9841) are indicated by white, black, and grey bars, respectively. The indole NH proton of Trp71 is indicated by 71s.

The same experiments are shown in Figures 2d, 2e and 2f respectively. The data in Figure 2d is fitted to  $k_{ex}^{obs}(+G) = Ck_{ex}^{obs}$ , where  $C$  is a factor by which EX2

- 30 mechanism exchange is catalysed by GroEL fragment. The values of  $C$  for the

globally, locally, and mixed amide protons of barnase are 10, 5, and 1. The data in Figures 2e and 2f is fitted to  $k_{\text{ex}}^{\text{obs}}(+G) = Ck_{\text{ex}}^{\text{obs}} + k_{\text{ex}}^{\text{obs}}(\text{G.U})$ , where  $k_{\text{ex}}^{\text{obs}}(\text{G.U})$  is the observed EX1 rate constant for global exchange of barnase in the presence of GroEL fragment. Figures 2d, 2e and 2f serve to demonstrate a mechanism of amide proton exchange of barnase. In Fig 2a and 2d there is only an estimate of the final concentration of GroEL fragment. At the high initial protein concentration used, GroEL 191-345 tended to crystallise during the exchange experiment.

Figures 2a, 2b and 2c show that the GroEL fragments catalyse, under folding conditions, exchange of amide protons of native barnase that are known to exchange only from its fully unfolded state (Perrett *et al* (1995) *Biochemistry* **34**: 9288-9298). Thus, like intact GroEL (Zahn *et al* (1996) *Science* **271**: 642-645), the apical domain binds with high affinity to unfolded barnase, and helices H11 and H12 are not essential for polypeptide binding (Figures 2a and 2b). The presence of the N-terminal histidine-tail does not abolish binding activity (Figures 2b and 2c).

The mechanism of exchange at pD 6.6 is EX2 (Figure 2d), but changes to EX1 at higher pD (Figures 2e and 2f). In an EX2 mechanism (Hvidt A and Nielsen S O (1966) *Advan Protein Chem* **21**: 287-386), the observed rate constant is limited by the intrinsic rate constant for exchange, which depends on pD. The criterion for an EX2 mechanism is that the rate constant for reprotection (ie a refolding or dissociation reaction) is much greater than the intrinsic rate constant. At the other extreme, when intrinsic exchange is much faster than reprotection, the mechanism becomes EX1 (Hvidt A and Nielsen S O (1966) *supra*) and the observed rate constant is simply equal to the rate constant for the formation of the unprotected state. Thus, from the exchange behaviour (and assuming that the presence of the histidine-tail does not effect binding) the rate constant for dissociation of barnase from the apical domain is about  $2 \text{ s}^{-1}$ , which is less than five-fold larger than that from intact GroEL (Zahn *et al* (1996) *supra*).



### Example 3 - Refolding of rhodanese

Rhodanese refolding assays are performed using GroEL, GroES, ATP, sht-GroEL 191-345 and sht-GroEL 191-376 and carried out as described by Horowitz  
5 (Horwitz P M in Protein Stability and Folding (eds Shirley B A) 361-368 (Humana Press, 1995)).

In more detail, rhodanese (9  $\mu$ M) is unfolded for 45 min at 25°C in the presence of 8 M urea and 1 mM  $\beta$ -mercaptoethanol. Refolding is initiated by diluting 3  $\mu$ l of  
10 the unfolded rhodanese into a final volume of 250  $\mu$ l of a standard buffer containing 50 mM Tris-HCl pH 7.8, 50 mM sodium thiosulphate, 10 mM  $MgCl_2$ , 10 mM KCl. GroEL, GroES, ATP, apical domain (or its C-truncated form) and bovine serum albumin (BSA) are included as indicated, at final concentrations of 2.5  $\mu$ M monomer, 2.5  $\mu$ M monomer, 2mM, 2.5  $\mu$ M and 45  $\mu$ g/ml (the same concentration  
15 by weights as the GroEL fragments), respectively.

After incubation for 50 min at 25°C, rhodanese activity is measured by adding 25  $\mu$ l from the refolding mixture to 1 ml of 50 mM  $Na_2S_2O_3$ , 50 mM KCN and 40 mM potassium phosphate buffer pH 8.6. The reaction is terminated after 15 min  
20 incubation by addition of 0.5 ml of 18% formaldehyde. Colour is developed by mixing with 1.5 ml of ferric nitrate reagent (400 g  $FeNO_3 \cdot 9H_2O$ ; 800 ml 65%  $HNO_3$  in a final volume of 3 dm<sup>3</sup>) prepared as indicated (Perrett *et al* (1995) *supra*).

Figure 3a shows the relative enzymatic activity of rhodanese (0.1  $\mu$ M) after  
25 refolding in the presence (+) or absence (-) of GroEL (2.5  $\mu$ M monomer), GroES (2.5  $\mu$ M monomer), ATP (2 mM), sht-GroEL 191-345 (2.5  $\mu$ M), sht-GroEL 191-376 (2.5  $\mu$ M), or bovine serum albumin (BSA; 45  $\mu$ g/ml), from 8 M urea (U). 100% activity is obtained with native rhodanese (N) alone.

Figure 3b shows the refolding kinetics of rhodanese in presence GroEL, GroES, and ATP (•). The final concentrations are the same as in Figure 2a. 100% activity is obtained with native rhodanese (O).

- 5 Figures 3c and 3d show the refolding kinetics of rhodanese in the presence of 0.18  $\mu$ M ( ), 2.5  $\mu$ M (•), or 5  $\mu$ M (O) sht-GroEL 191-345 and sht-GroEL 191-376, respectively.

Figure 3a shows a rhodanese refolding activity of about 42.5% for shtGroEL 191-345 which is about 37.5% above background refolding as shown by the control of unfolded rhodanese (U) alone.

The results of Figure 3b are for comparative purposes and show a time course of refolding activity for GroEL, GroES and ATP.

15

Figure 3c shows the rhodanese refolding kinetics at various concentrations of sht-GroEL 191-345. Refolding activities of about 50% are achieved at about 25 mins.

Figure 3d is similar to Figure 3c but for sht-GroEL 191-376 and showing refolding activities of about 40% after 25 mins.

Enzymatic activity is obtained from the absorbance at 460 nm of the complex formed between thiocyanate and ferric ion. Results correspond to the average of three different independent assays. Standard error bars are shown. *b-d*, same as in *a*, but, to stop the refolding reaction rhodanese activity is assayed in the presence of 10 mM trans-1,2-cyclohexanediaminetetraacetate (CDTA) to inhibit GroEL activity or 0.5 mg/ml of casein to saturate the apical domain.

Maximal refolding yield is obtained at molar ratios of apical domain and rhodanese of larger than one, from which is estimated a dissociation equilibrium constant of  $>1 \times 10^{-7}$  M.

#### 5 Example 4 - Refolding of cyclophilin A

The chaperone activity of sht-GroEL 191-345 and sht-GroEL 191-376 is tested using cyclophilin A. Refolding of cyclophilin A is initiated by diluting 8 M urea denatured protein (100  $\mu$ M) into 100 mM potassium phosphate buffer pH 7.0, 10 mM DTT to a final concentration of 1  $\mu$ M. The final concentration of GroEL and apical domain in refolding buffer is 7  $\mu$ M and 4  $\mu$ M or 1  $\mu$ M, respectively. Refolding temperature is 25°C. After incubation for the times indicated, cyclophilin activity is measured as described (Fischer G *et al* (1984) Biomed Biochim Acta 43: 1101-1111). Spontaneous refolding of cyclophilin A occurred to a yield of about 15 30%, and is finished in less than 1 min. Standard error is 5%.

Figure 3e shows the refolding of 1  $\mu$ M cyclophilin A in the presence of 7  $\mu$ M GroEL monomer (O), 4  $\mu$ M sht-GroEL 191-345 (•), 4  $\mu$ M sht-GroEL 191-376 ( ), or 1  $\mu$ M sht-GroEL 191-376 ( ). 100% activity is obtained with native cyclophilin A. Figure 3e demonstrates that 100% refolding of inactivated cyclophilin A can be 20 achieved with sht-GroEL 191-345 or sht-GroE 191-376 and that this is equivalent to that seen with GroEL.

Cyclophilin A refolds only at low yield in the absence of chaperone but refolding is 25 facilitated in the presence of GroEL monomer owing to a transient complex formation (see Zahn *et al* (1996) FEBS Lett 380: 152-156). A similar rate constant is found for refolding of cyclophilin A in the presence of intact GroEL monomer and in the presence of GroEL fragments, which within a factor of four does not depend on chaperone concentration (Figure 3e). Maximal refolding yield is 30 obtained at stoichiometric concentrations of cyclophilin A and apical domain,

indicating the formation of 1:1 complex between chaperone fragment and substrate protein during refolding.

Example 5 - Thermal denaturation of sht-GroEL 191-376 and sht-GroEL 191-345

5

Thermal denaturation monitored by far ultra violet-circular dichroism (UV-CD) at 222 nm and is carried out on a Jasco J720 spectropolarimeter interfaced with a Neslab RTE-100 water bath, using a thermostated cuvette (Helma) with 1 mm path length. The temperature is increased at a linear rate of 50 deg/h. The protein concentration is 40  $\mu$ M in 10 mM sodium phosphate buffer pH 7.0. Data are fitted to a denaturation curve (Pace, C N (1990) Trends Biotechnol 8: 93-98) to determine  $T_m$ , the midpoint temperature of denaturation.

Thermal denaturation is also monitored by differential scanning calorimetry (DSC) and the measurements are performed at a protein concentration of  $88 \pm 5$   $\mu$ M in 10 mM sodium phosphate buffer pH 7.0, using a Microcal MC-2D instrument at a notional scan rate of 60 deg/h. Sample preparation and data analysis are performed as described previously (Johnson C M and Fersht A R (1995) Biochemistry 34: 6795-6804). Both proteins exhibit at least 50% reversibility in their thermal unfolding as judged from the area of endotherms obtained on rescanning samples. Higher levels of reversibility are obtained at lower concentrations or by stopping scans at temperatures closer to the main unfolding transition. The low temperature transition observed in sht-GroEL 191-376 is completely reversible with scans limited in temperatures below 50°C.

25

Figure 4a shows the far UV-CD results and Figure 4b shows the DSC results. In each Figure, sht-GroEL 191-376 is the upper trace and sht-GroEL 191-345 is the lower trace.

The C-terminal helices are found to be flexible. The apical domain and the fragment truncated at position 345 are reversibly denatured by temperature or urea, and the denaturation is not influenced by the N-terminal histidine-tail. There are two cooperative folding transitions, at 34°C and 67°C. At 45°C, the CD spectrum of the apical domain is identical to that of the truncated domain. The C-terminal  $\alpha$ -helices, therefore, must melt at the lower temperature and separately from the "domain core". The second cooperative transition associated with the extra 31 amino acids in sht-GroEL 191-376 is confirmed by DSC (Figure 4b). The calorimetric data are also essentially consistent with the unfolding of the apical domain as a monomer. At physiological temperature, about 50% of helices H11 and H12 are in an unfolded conformation, and thus flexible.

#### Example 6 - Crystallisation of GroEL fragments and diffraction study

Crystals are obtained from hanging drops initially containing sht-GroEL 191-345 at 23 mg ml<sup>-1</sup>, 11% PEG 4000, 50 mM Tris-HCl pH 8.5 and 100 mM LiSO<sub>4</sub>, equilibrated against reservoirs consisting of 22% PEG 4000, 100 mM Tris-HCl pH 8.5 and 200 mM LiSO<sub>4</sub>. X-ray data are collected from a capillary-mounted crystal at 4°C using a 30 cm Mar Research image plate detector at station 9.6 of the Synchrotron Radiation Source (SRS) at Daresbury, UK ( $\lambda=0.87$  Å). Unless stated otherwise, all data processing, data reduction, electron density syntheses and structural analyses are carried out using CCP4 software (Daresbury Laboratory, Warrington, UK). Indexing and intensity measurements of diffraction data are carried out with the MOSFLM program suite Leslie A G W in Joint CCP4 and ESF-EACMB Newsletter on Protein Crystallography No 26 (Daresbury Laboratory, Warrington, UK, 1992).

The structure is solved by conventional molecular replacement methods, using the program AMORE (Navaza J (1994) *Acta Crystallogr A* 50: 157-163), and a search model consisting of residues 191-345 of the refined structure of GroEL (Braig *et al*

(1995) *Nature Struct Biol* 2: 1083-1094). The asymmetric unit contains one protein molecule, corresponding to a solvent content of 51%.

Model rebuilding and refinement is carried out with O (Jones *et al* (1991) *Acta Crystallogr A* 47: 110-119), and the structure is refined using X-PLOR (Brünger A T X-PLOR, Version 3.1, A System for Crystallography and NMR (Yale Univ Press, New Haven, CT, 1992)), using Engh and Huber parameters (Engh R A & Huber R (1991) *Acta Crystallogr A* 47: 392-400). The current model contains 8 water molecules and is complete with the following exceptions, which could not be modelled due to poor or non-existent electron density: residues 302-307 and residues 337-345 from the C-terminus. Electron density for the N-terminal His-tag is also not observed. No residues have disallowed backbone  $\phi\psi$  angles. Table 1 provides a summary of the crystallographic data.

TABLE 1 Summary of crystallographic data

*Data collection statistics*

Unit cell dimensions	$a = b = 91.67 \text{ \AA}, c = 38.33 \text{ \AA}$
Space group	$P3_121$
Resolution ( $\text{\AA}$ )	22.0-2.5
Measured reflections	21,762
Unique reflections	6,564
Completeness of data (%) <sup>*</sup>	99.4 (96.7)
$R_{\text{merge}}$ (%) <sup>*†</sup>	9.9 (45.1)
$\langle F/\sigma F \rangle$ <sup>*</sup>	19.8 (4.6)
Multiplicity <sup>*</sup>	3.3 (3.0)

*Refinement statistics*

Resolution ( $\text{\AA}$ )	8.0-2.5
$R$ -factor/free $R$ -factor (%), $F > 0^\ddagger$	21.4/29.1
r.m.s.d bond length ( $\text{\AA}$ )	0.006
r.m.s.d bond angle (deg)	1.42

\* values given in parenthesis are for the highest resolution shell.

† agreement between intensities of repeated measurements of the same reflections and can be defined as:  $\Sigma(I_{h,i} - \langle I_h \rangle) / \Sigma I_{h,i}$ , where  $I_{h,i}$  are individual values and  $\langle I_h \rangle$

5 is the mean value of the intensity of reflection  $h$

‡ The free R-factor is calculated with the 10% data omitted from the refinement (test set, prepared using DATAMAN (Kleywegt, G.J. & Jones, t.A. Acta. crystallogr. D 50, 178-185 (1994)

## 10 Example 7 - The three dimensional structure of sht-GroEL 191-345

Figure 5a shows the secondary structure representation drawn with MolScript (Kraulis P J (1991) J Appl Crystallogr 24: 946-950) and Raster3D (Merrit E A and Murphy M E P (1994) Acta Crystallogr D 50: 869-873). Helices are labelled as in  
15 Braig *et al* (Braig *et al* (1995) Nature Struct Biol 2: 1083-1094). N and C refer to the N-terminus (residue 191) and C-terminus (residue 336) of the model.

Figure 5b is a backbone representation drawn with program O (Jones *et al* (1991) Acta Crystallogr A 47: 110-119), in same orientations as Figure 5a colour-coded  
20 according to B-factor of main-chain atoms: blue ( $20 \text{ \AA}^2$ ) to red ( $60 \text{ \AA}^2$ ).

Figure 5c shows a representative region of electron density, calculated using refined co-ordinates, viewed along the helices H8 and H9.

25 Crystals of sht-GroEL 191-345 grow in the trigonal space group  $P3_121$  with one molecule per asymmetric unit, giving a solvent content of 51%. The three dimensional structure of the "domain core" is solved by molecular replacement and refined to an R-factor of 21.4% and a free R-factor of 29.1% for all data between 8.0 and  $2.5 \text{ \AA}$  (Table 1). The quality of the electron density map is shown in  
30 Figure 5c.

Overall, sht-GroEL 191-345 has the same fold as the corresponding region of the intact GroEL protein (Figure 5a): two orthogonal  $\beta$ -sheets forming a  $\beta$ -sandwich, flanked by three  $\alpha$ -helices. The structure is more ordered and better resolved than is the apical domain of the intact protein (Figure 5b): the average  $B$ -factor is 42  $\text{\AA}^2$ , compared with 97  $\text{\AA}^2$  for residues 191-336 of the GroEL structure. Unfortunately, such unusually high disorder in the GroEL structure complicates the interpretation of a structural comparison, in the same way as it can be misleading to use an average of NMR structures for comparison with a crystal structure. and so we have not done so here. In essence, the structure can be described as a well ordered  $\beta$ -sandwich scaffold, flanked by relatively flexible helical and loop regions. In particular, the  $B$ -factors of most of the  $\beta$ -strand,  $\alpha$ -helix, and loop structure is about 20, 40, and 60  $\text{\AA}^2$ , respectively (Figure 5b). There are two regions of considerable disorder. First, electron density is not found for the C-terminal residues 337-345, which correspond to the first half of  $\alpha$ -helix H11. This is an agreement with the results from folding experiments, described above. Second, electron density for residues 302-307 is very poor and fragmented. This region is part of the most disordered segment in the structure of intact GroEL (Braig *et al* (1995) Nature Struct Biol 2: 1083-1094). The content of  $\alpha$ -helix and  $\beta$ -sheet secondary structure of the GroEL fragment (Figure 1) is 48% and 74% higher, respectively, compared with the corresponding region of intact GroEL. There are four additional segments of secondary structure in the new structure (Figures 1 and 5a): residues 299-301 form a short  $\beta$ -strand; residues 201-205, 229-232 and 308-317 form  $3_{10}$ -helices.

25 Example 8 - Refolding chromatography of IGPS (49-252) (indoleglycerol phosphate synthase lacking residues 1-48)

The fragments GroEL(191-345) or GroEL(191-376) are expressed in *E. coli* with a 17-residue N-terminal tail containing 6 histidine residues so that they could be purified using Ni-NTA resin - the  $\text{Ni}^{2+}$  ion which is chelated by the agarose binds



to the histidine tag. Initially, the same resin is used to immobilise purified GroEL(191-345) or GroEL(191-376) by their histidine tags for preparative purposes. Subsequently, the purified fragments are also linked covalently to agarose via CNBr activation (Axen R *et al* (1967) Nature 1302-1304). The  
5 immobilised GroEL fragments are found to facilitate the refolding of cyclophilin A with high efficiency.

The apical domain of GroEL (GroEL(191-376)) and the "core" of the apical domain, GroEL(191-345), are cloned and expressed in *E. coli* as fusion proteins  
10 containing a 17-residue N-terminal histidine-tail. The fragments are immobilised by two methods.

*A). Immobilised apical domain attached to Ni-NTA resin.*

The Ni-NTA resin (from QIAGEN) is a chelating adsorbant composed of a high  
15 surface concentration of nitrilo-tri-acetic acid (NTA) ligand attached to Sepharose CL-6B. The NTA occupies four of six ligand binding sites in the coordination sphere of the  $\text{Ni}^{2+}$  ion, leaving two sites free to interact with the six histidines in the N-terminal tail. Proteins containing one or more 6 x His affinity tags, located at either the amino or carboxyl terminus of the protein, bind to the Ni-NTA resin with  
20 an affinity ( $K_d = 10^{-13}$  M, pH 7.8). The stability of the 6 x His/Ni-NTA interaction is unaffected by strong denaturants such as 6M guanidine hydrochloride or 8M urea, or the presence of low levels of  $\beta$ -mercaptoethanol (1-10 mM). 3.5 mL of Ni-NTA resin are equilibrated with 0.1M potassium phosphate at pH 7.8, containing 5 mM  $\beta$ -mercaptoethanol. The GroEL domain is added to saturation of the affinity  
25 gel (21 mg of protein per 3.5 mL of gel) and incubated at room temperature for 30 min with gentle mixing. The gel is packed in a column suitable for FPLC (5 x 100 mm, from Pharmacia) and thoroughly washed with the initial buffer.

*B). Immobilised apical domain attached to CNBr-activated Sepharose 4B*

- To minimise the steric effects and preserve the structure of the binding site in the apical domain, the binding capacity of the gel is reduced by controlled hydrolysis of the activated gel before coupling. 300 mg of freeze-dried powder are suspended in
- 5 50 mM of  $\text{NaHCO}_3$  pH 8.3, washed with the same buffer and re-swollen on a sintered glass filter (G3), then suspended in the buffer and mixed in an end-over-end shaker for 4 h at room temperature. The apical domain, dissolved in the coupling buffer (0.1M  $\text{NaHCO}_3$ , pH 8.3 and 0.5 M NaCl), is added to the gel suspension (10 mg protein/mL gel) and mixed in an end-over-end shaker for 6 h at
- 10 room temperature. It is then washed with the coupling buffer. The remaining active groups are blocked by adding 2.5 M ethanolamine pH 8 and shaking for 4 h at room temperature. Uncoupled apical domain is removed by washing with five cycles of alternately high and low pH buffer solution (Tris-HCl 0.1M pH 7.8 containing 0.5M NaCl followed by acetate buffer (0.1M, pH 4 plus 0.5M NaCl).
- 15 The gel is finally washed with 0.1 M potassium phosphate at pH 7.8, containing 5 mM 2-mercaptoethanol (refolding buffer).

*C). Immobilised apical domain attached to NHS-Sepharose*

- Ligand coupling. NHS-activated sepharose ( 5 ml ) is unpacked from a HiTrap
- 20 NHS-activated column and washed extensively (50 ml) with 1 mM HCl, ice cold, using a Buchener funnel. The binding solution (mini-chaperone) can then be added to the slurry in the desired concentration (10mg/ml).

- Binding solution: minichaperone (10mg/ml), in  $\text{NaHCO}_3$  pH 9, 0.5M NaCl, is
- 25 gently mixed and then a saturated solution of sodium sulfate is added, until it just starts to become cloudy (just before the concentration reaches the precipitation point). When reacting the support, it is necessary to gently agitate the reaction tubes by tumbling for 20 hrs at room temperature. Once immobilization and any quenching reactions are complete, the support is washed extensively first with

coupling buffer, then with a high salt (1M NaCl) buffer to eliminate ligand that may be bound to the support through protein/protein interactions.

Excess groups are blocked with 1M ethanolamine for at least 4 hr or overnight, at  
5 RT, then the Sepharose is washed with bicarbonate buffer (0.1M, pH 9.2) and acetate buffer (0.1M pH 4.0), before a final wash in refolding buffer.

The immobilised fragments are used in a chromatography column (Figure 6). Denatured protein in urea is added to the column and developed with a refolding  
10 buffer. The protein eluted as from a conventional binding column, its passage is retarded and the peak could be characterised by a retention time. Some mutants of indoleglycerol phosphate synthase (IGPS) are expressed *E. coli* and isolated as inclusion bodies. These reprecipitate on attempts to renature them after dissolving  
15 at high concentrations in urea, and attempts at low protein concentrations yielded soluble material of non-native conformation. The mutant IGPS (49-252) (1 x 22 kDa), which lacks the first 48 amino acid residues, is obtained on chromatography on the GroEL(191-345) column in a 92% yield of material that have 100% binding activity (Figure 6). The term "refolding chromatography" can be used to describe the phenomenon of refolding by passage through the column.

20

In more detail, a column of ht-GroEL 191-345 immobilised on Ni-NTA agarose (3.0 mL) is loaded with 2 nmol of IGPS (49-252) dissolved in 20  $\mu$ L of 8 M urea, and the column is developed with refolding buffer (0.1 M potassium phosphate at pH 7.8, containing 5 mM 2-mercaptoethanol) using a Waters 625 LC HPLC  
25 system. In Figure 6, the two peaks are seen which are described by:  $K_{av} = (V_e - V_0)/(V_t - V_0)$ , where  $V_t$  is the total volume of gel in the column,  $V_0$ , the void volume, and  $V_e$  the volume at the maximum of the peak. A value of  $K_{av}$  indicates that the protein interacts with the support. Peak 1 have  $K_{av} = 1.0$  (9.6 % of the total area) and peak 2  $K_{av} = 1.7$  (90.4%). The protein in peak 2 is recovered in  
30 1.2 mL, and contained 1.85 nmol of truncated IGPS (92.5 % yield). It displays a

circular dichroism spectrum characteristic of a native a/b protein and bound  $^3\text{H}$ -rCdRP (tritium-labelled reduced 1[(2-carboxyphenyl)amino]-1-deoxyribulose 5-phosphate), a specific inhibitor of IGPS, with a stoichiometry of 1.0. Initially denatured cyclophilin chromatographs with two peaks, an inactive at  $K_{av} = 0.38$  and an active at  $K_{av} 1.43$

#### Example 9 - Batchwise renaturation of cyclophilin A on Ni-NTA agarose gels

This experiment uses batchwise mixing of materials. A solution of denatured cyclophilin in 8 M urea is diluted 100 fold with Ni-NTA-immobilised GroEL(191-345) in a refolding buffer (see Table II below). After gentle mixing for 30 min, the cyclophilin is recovered in 84% yield of protein. Control experiments (Table II) in which the protein is denatured in 8 M urea and added to the refolding buffer alone, or mixed with agarose that have not been linked to GroEL fragments, gave cyclophilin that have only 20% of the activity of the starting material before denaturation. The cyclophilin used for the denaturation experiments have 88% of the activity of the purest samples previously obtained by the inventors. The specific activity of the material obtained from the GroEL(191-345)-agarose resin (covalently linked) is 126% of that of the previous purest samples. Further, the total recovery of activity is 25% more than that initially present. Thus, the immobilised GroEL fragment have "reconditioned" the cyclophilin A by converting inactive material into active.

In more detail, suspension of 200  $\mu\text{L}$  of gel (wet, sedimented volume) is mixed with refolding buffer (100 mM phosphate buffer, pH 7.8 plus 5 mM 2-mercaptoethanol) to give a volume of 990  $\mu\text{L}$ . 10  $\mu\text{L}$  of cyclophilin (from a 100  $\mu\text{M}$  stock solution in refolding buffer + 8M urea, = 1 nmol of cyclophilin A) are added and the suspension is mixed in an up-down mixer for 30 min at room temperature.

The gel suspension is centrifuged to separate the supernatant ( $\sim 800 \mu\text{L}$ ). The gel pellet is washed in miniprep columns and the eluate added to the supernatant to give about  $900 \mu\text{L}$ . The protein concentration is determined from the  $A_{280 \text{ nm}}$ . Cyclophilin activity is measured in the supernatant as described in (Makino Y *et al*  
5 (1993) FEBS Lett 336: 363-367). The sample prior to denaturation have 88% of the specific activity of the highest activity of native cyclophilin previously obtained by the inventors. The control is agarose alone, without Ni. The results are shown in table II below.

Table II

Gel type	Protein yield	Cyclophilin activity yield	Cyclophilin specific activity relative to "pure" native
	(% )	(% )	(% )
GroEL 191-345 agarose	84	98	103
GroEL 191-376 agarose	81	105	115
GroEL 191-345 agarose	87	125	126
Agarose (control) <sup>5</sup>	72	17	20
15 Native cyclophilin (control)			88

The procedure is applied to other proteins used in the laboratory. Glucosamine 6-phosphate deaminase (6 x 30 kDa) (Oliva G *et al* (1995) Structure 3: 1323-1332) normally regains only 10 % or less activity after renaturation from urea denaturation. A 100 % yield is obtained on batchwise treatment with GroEL(191-345)-agarose. Further, a sample that have lost all activity on storage in solution in 50% glycerol/water at -20 °C for 5 years also regained 100 % activity with this treatment after dissolving in urea.

#### Example 10 - Residues of GroEL implicated in binding peptides

10

The X-ray crystal structure of GroEL 191-376 with the 17 residue N-terminal tail shows that seven residues of the tail of one molecule bind in the active site of the other. Residues 230-271 are in the binding site. All residues are shown in Table III in which large, bold and underlined residues are those detected by X-ray crystal structure of ht GroEL 191-376 as being involved in protein binding.

15

The X-ray crystal structure shows that 193-336 should be reasonably stable. The 193-337 fragment is cloned and expressed and found to be stable. Therefore, residues 191 and 192 may be omitted.

20

Table III

---

	190	VEGMQFDRGY	LSPYFINKPE	TGAVELESPF	ILLADKKISN
25	230	<b>I</b> RE <b>M</b> <b>L</b> PV <b>L</b> EA	V <b>A</b> KAGKPLLI	IAEDVEGEAL	<b>A</b> T <b>L</b> V <b>V</b> N <b>T</b> M <b>R</b> G
	270	<b>I</b> V <b>K</b> VAAVKAP	GFGDRRKAML	QDIATLTGGT	VISEEIGMEL
	310	EKATLEDLGQ	AKRVVINKDT	TTIIDGVGEE	AAIQGRVAQI

---

Example 11 - Construction and testing of minimal minichaperone fragments

Various N and C terminally truncated fragments of the apical domain of GroEL, including fragment 193-335, are cloned by PCR into *Bam*HI and *Eco*RI sites of the  
5 pRSET A vector (Invitrogen) encoding an N-terminal histidine tail (17 amino acids; "sht") which comprises an engineered thrombin cleavage site (Zahn. *et al.*, (1996) PNAS (USA) 93:15024-15029). Plasmid construction is verified by PCR cycle sequencing using fluorescent dideoxy chain terminators according to the manufacturer's instructions (Applied Biosystems). Sequencing reactions are  
10 analysed on an Applied Biosystems 373A Automated DNA Sequencer.

The overexpression and purification of the minichaperone in *E. coli* BLR(DE3) cells is carried out essentially as previously described (Zahn *et al.*, *Supra*). sht-GroEL(193-335) is over-expressed in BLR(DE3) cells to give ~100 mg purified  
15 protein per litre of culture. The GroEL fragments are analysed by quantitative amino acid analysis, N-terminal sequencing, and mass spectrometry. Protein concentration is determined by absorbance at 276 nm using an extinction coefficient calculated from Gill & von Hippel, (1989) Anal. Biochem. 182:319-326. In order to assess the degree of association between the fragments, dynamic light scattering  
20 experiments are performed at 25°C with a DynaPro-801TC Molecular Sizing instrument (Protein Solutions, Inc., Charlottesville, VA, USA). The protein concentrations used are in the  $\mu$ M range in 50 mM Tris-HCl, 150 mM NaCl, pH 8.2 buffer. sht-GroEL(193-335) purified by gel-filtration is monomeric at  $\mu$ M concentrations, as determined by light scattering experiments.



Refolding assays of rhodanese and cyclophilin A are performed as above. sht-GroEL 193-335 is as active *in vitro* as sht-GroEL(191-345) and sht-GroEL(191-376) in chaperoning the folding of rhodanese and cyclophilin A.

5

Example 12 - Alignment of consensus binding sequences of cpn60 family members

Figure 9 shows sequences in OWL database release 28.1 containing clear homology to apical domain of GroEL (residues 191-375) in PDB structure pdb1grl.ent. OWL is a non redundant database merging SWISS-PROT, PIR (1-3), GenBank (translation) and NRL-3D. Consensus sequence = residues 230-271 inclusive) containing peptide-binding site (as identified by crystal structure analysis and polypeptide binding studies). X = residue in peptide-binding site in the X-ray crystal structure of mini chaperone. The GroEL *E. coli* chaperone sequence is shown in italics.

## Claims:

1. A chaperone polypeptide having an amino acid sequence selected from at least amino acid residues 230-271 but no more than residues 150-455 or 151-456 of a GroEL sequence substantially as shown in Figure 7, or a corresponding sequence of a substantially homologous chaperone polypeptide, or a modified, mutated or variant sequence thereof having chaperone activity.

2. Monomeric polypeptide having chaperone activity and incapable of multimerisation in solution.

3. A chaperone polypeptide which, when in solution, remains monomeric and has the ability to refold, reactivate or recondition proteins, said polypeptide including the protein binding active site motif:

1 X X X 2 X X 3 4 X X 5 X X X X X X X X X X X X X X 6 X X 7  
8 X 9 10 11 X X 12 X 13 14

wherein 1 is selected from amino acid residues:

1. M, L, V, S, F or A;

wherein 2 is selected from: L, I, P, V or A;

wherein 3 is selected from: L, E, V, H or I;

wherein 4 is selected from: E, A, R, L, Q, or N;

wherein 5 is selected from: A, V, I, M, L, N, S, R, T, Q or K;

wherein 6 is selected from: E, D or G;

wherein 7 is selected from: A, P, S, T, G or L;

wherein 8 is selected from: T, A, N, S or V;

wherein 9 is selected from: V, L, I or A;

wherein 10 is selected from: V, L, I, F or H;

wherein 11 is selected from: N, S or L;

wherein 12 is selected from: R, K, N, Q, L or S;

wherein 13 is selected from: I, T, S, G, V, A, Q, N, K, F or P;

wherein 14 is selected from: V, I, L, F, D or T; and

5 wherein the Xs represent a peptide bond or bonds or at least one amino acid residue,

or a functional variant thereof in which one or more of the numbered amino acid residues 1 to 14 has undergone a conservative substitution.

10

4. A chaperone polypeptide which, when in solution, remains monomeric and has the ability to refold, reactivate or recondition proteins, said polypeptide including at least one protein binding active site motif moiety selected from:

15 (a) 1 X X X 2 X X 3 4 X and

(b) X 5 X X X X X X X X X X X X X X 6 X X 7 8 X 9 10  
11 X X 12 X 13 14

20 wherein 1 is selected from amino acid residues:

I, M, L, V, S, F or A;

wherein 2 is selected from: L, I, P, V or A;

wherein 3 is selected from: L, E, V, H or I;

wherein 4 is selected from: E, A, R, L, Q, or N;

25 wherein 5 is selected from: A, V, I, M, L, N, S, R, T, Q or K;

wherein 6 is selected from: E, D or G;

wherein 7 is selected from: A, P, S, T, G or L;

wherein 8 is selected from: T, A, N, S or V;

wherein 9 is selected from: V, L, I or A;

30 wherein 10 is selected from: V, L, I, F or H;

wherein 11 is selected from: N, S or L;  
wherein 12 is selected from: R, K, N, Q, L or S;  
wherein 13 is selected from: I, T, S, G, V, A, Q, N, K, F or P;  
wherein 14 is selected from: V, I, L, F, D or T; and

5

wherein X is at least one amino acid residue,

or a functional variant thereof in which one or more of the numbered amino acid residues 1 to 14 has undergone a conservative substitution.

10

5. A chaperone polypeptide which, when in solution, remains monomeric and has the ability to refold, reactivate or recondition proteins, said polypeptide including the protein binding active site motif:

15 I X X X L X X L E X X A X X X X X X X X X X X X X X X E X X  
A T X V V N X X R X I V

20

wherein X is at least one amino acid residue, or a functional variant thereof in which one or more of the specified amino acid residues has undergone a conservative substitution.

25

(a) I X X X L X X L E X

(b) X A X X X X X X X X X X X X X X X E X X A T X V V  
N X X R X I V

30

wherein X is at least one amino acid residue, or a functional variant thereof in which one or more of the specified amino acid residues has undergone a conservative substitution.

- 5 7. A chaperone polypeptide as claimed in any one of claims 3 to 6 in which the active site motif or an active site motif moiety includes the conserved sequence:

PLL(V)I(V)IA(S)EDV(I)EGEAL

- 10 in which amino acid symbols in parenthesis are alternatives to the immediately preceding symbol reading left to right.

8. Monomeric polypeptide having chaperone activity and incapable of multimerisation characterised in that in the absence of ATP the polypeptide has a  
15 protein refolding activity of more than 50%, preferably 60%, even more preferably 75%, said refolding activity being determined by contacting the polypeptide with an inactivated protein of known specific activity prior to inactivation, and then determining the specific activity of the said protein after contact with the polypeptide, the % refolding activity being:

20

$$\frac{\text{specific activity of protein after contact with polypeptide} \times 100}{\text{specific activity of protein prior to inactivation}} \quad 1$$

- 25 9. A polypeptide as claimed in any preceding claim, wherein the chaperone activity is determined by the refolding of cyclophilin A.

10. A polypeptide as claimed in claim 9 wherein 8M urea denatured cyclophilin A (100μM) is diluted into 100mM potassium phosphate buffer pH7.0, 10mM DTT  
30 to a final concentration of 1μM and then contacted with at least 1μM of said polypeptide at 25°C for at least 5 min, the resultant cyclophilin A activity being

assayed by the method of Fischer G *et al* (1984) Biomed Biochim Acta 43: 1101-1111.

11. A polypeptide as claimed in any preceding claim being an hsp60  
5 polypeptide, preferably a GroEL polypeptide.
12. A polypeptide as claimed in any preceding claim which comprises at least an amino acid sequence selected from GroEL residues:
  - 10 (a) 191-329, 191-330, 191-331, 191-332, 191-333, 191-334, 191-335, 191-336, 191-337, 191-338, 191-339, 191-340, 191-341, 191-342, 191-343, 191-344, 191-345, 191-346, 191-347, 191-348, 191-349, 191-350, 191-351, 191-352, 191-353, 191-354, 191-355, 191-356, 191-357, 191-358, 191-359, 191-360, 191-361, 191-362, 191-363, 191-364, 191-365, 191-366, 191-367, 191-368, 15 191-369, 191-370, 191-371, 191-372, 191-373, 191-374, 191-375 or 191-376, or
  - (b) 192-329, 192-330, 192-331, 192-332, 192-333, 192-334, 192-335, 192-336, 192-337, 192-338, 192-339, 192-340, 192-341, 192-342, 192-343, 192-344, 20 192-345, 192-346, 192-347, 192-348, 192-349, 192-350, 192-351, 192-352, 192-353, 192-354, 192-355, 192-356, 192-357, 192-358, 192-359, 192-360, 192-361, 192-362, 192-363, 192-364, 192-365, 192-366, 192-367, 192-368, 192-369, 192-370, 192-371, 192-372, 192-373, 192-374, 192-375 or 192-376, or
  - 25 (c) 193-329, 193-330, 193-331, 193-332, 193-333, 193-334, 193-335, 193-336, 193-337, 193-338, 193-339, 193-340, 193-341, 193-342, 193-343, 193-344, 193-345, 193-346, 193-347, 193-348, 193-349, 193-350, 193-351, 193-352, 193-353, 193-354, 193-355, 193-356, 193-357, 193-358, 193-359, 193-360, 30 193-361, 193-362, 193-363, 193-364, 193-365, 193-366, 193-367, 193-368,

193-369, 193-370, 193-371, 193-372, 193-373, 193-374, 193-375 or 193-376, or

- 5 (d) 230-271, 229-271, 229-272, 228-272, 228-273, ...*et seq.* 194-328, 194-329, or

the equivalent residues of substantially homologous chaperonins, or a modified, mutated or variant sequence thereof.

- 10 13. A polypeptide as claimed in claim 8, wherein the selected amino acid sequence is selected from the group consisting of 230-271, 191-345, 191-376, 193-335 and 193-337 of GroEL, the equivalent residues of substantially homologous chaperonins, and a modified, mutated or variant sequence thereof.

- 15 14. A polypeptide as claimed in any preceding claim further comprising a polyamino acid sequence, preferably an N-terminal polyamino acid sequence.

15. A polypeptide as claimed in claim 14, wherein the polyamino acid sequence is a polyhistidine sequence.

20

16. A polypeptide as claimed in claim 14 or claim 15, wherein the polyamino acid sequence includes a cleavage site cleavable by a cleavage agent, preferably said cleavage agent is thrombin.

- 25 17. A polypeptide as claimed in any one of claims 14 to 16 wherein the further polyamino acid sequence comprises a number of amino acid residues in the range 2 to 500, preferably 5 to 100, more preferably 17 to 39.

- 30 18. A polypeptide as claimed in any preceding claim and is immobilised form, optionally immobilised to a chromatographic matrix, preferably an agarose resin.

19. A polypeptide as claimed in claim 18, wherein the agarose resin is a nickel-nitrilo-tri-acetic acid (NTA)-ligated agarose resin.
- 5 20. A polypeptide as claimed in any preceding claim fused to a heterologous protein or polypeptide.
21. A recombinant polypeptide as claimed in any preceding claim.
- 10 22. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide as defined in any one of claims 1 to 21 or a nucleotide sequence hybridisable thereto and optionally encoding a polypeptide having chaperone activity.
- 15 23. A recombinant nucleic acid molecule for use in cloning and/or expressing a nucleic acid sequence, said recombinant nucleic acid molecule comprising:
- (a) a nucleotide sequence encoding amino acid residues 191-376 of GroEL, or
- 20 (b) a nucleotide sequence encoding amino acid residues 191-345 of GroEL, or
- (c) a nucleotide sequence encoding amino acid residues 193-337 of Gro EL, or
- (d) a nucleotide sequence encoding amino acid residues 193-335 of Gro EL, or
- 25 (e) a nucleotide sequence encoding amino acid residues of GroEL selected from amino acid residues:
- (i) 191-329, 191-330, 191-331, 191-332, 191-333, 191-334, 191-335, 191-336,
- 30 191-337, 191-338, 191-339, 191-340, 191-341, 191-342, 191-343, 191-344,



- 191-345, 191-346, 191-347, 191-348, 191-349, 191-350, 191-351, 191-352,  
191-353, 191-354, 191-355, 191-356, 191-357, 191-358, 191-359, 191-360,  
191-361, 191-362, 191-363, 191-364, 191-365, 191-366, 191-367, 191-368,  
191-369, 191-370, 191-371, 191-372, 191-373, 191-374, 191-375 or 191-  
5 376, or
- (ii) 192-329, 192-330, 192-331, 192-332, 192-333, 192-334, 192-335, 192-336,  
192-337, 192-338, 192-339, 192-340, 192-341, 192-342, 192-343, 192-344,  
192-345, 192-346, 192-347, 192-348, 192-349, 192-350, 192-351, 192-352,  
10 192-353, 192-354, 192-355, 192-356, 192-357, 192-358, 192-359, 192-360,  
192-361, 192-362, 192-363, 192-364, 192-365, 192-366, 192-367, 192-368,  
192-369, 192-370, 192-371, 192-372, 192-373, 192-374, 192-375 or 192-  
376, or
- 15 (iii) 193-329, 193-330, 193-331, 193-332, 193-333, 193-334, 193-335, 193-336,  
193-337, 193-338, 193-339, 193-340, 193-341, 193-342, 193-343, 193-344,  
193-345, 193-346, 193-347, 193-348, 193-349, 193-350, 193-351, 193-352,  
193-353, 193-354, 193-355, 193-356, 193-357, 193-358, 193-359, 193-360,  
193-361, 193-362, 193-363, 193-364, 193-365, 193-366, 193-367, 193-368,  
20 193-369, 193-370, 193-371, 193-372, 193-373, 193-374, 193-375 or 193-  
376, or
- (f) 230-271, 229-271, 229-272, 228-272, 228-273, ...*et seq.*... 194-328, 194-  
329, or
- 25 (g) a nucleotide sequence hybridisable to any of (a), (b), (c), (d), (e) or (f) above  
and encoding a monomeric polypeptide having chaperone activity, or
- (h) degenerate nucleotide sequences corresponding to (a), (b), (c), (d), (e), (f) or  
30 (g) above.

24. A vector comprising a nucleic acid as claimed in claim 22 or claim 23.
25. A host cell transformed with a nucleic acid as defined in claim 22 or claim  
5 23 or vector as defined in claim 24.
26. A method of making a polypeptide as defined in any one of claims 1 to 21  
comprising transforming a host cell with a nucleic acid encoding said polypeptide,  
culturing the transformed cell and expressing said polypeptide.
- 10 27. A method of making a polypeptide as claimed in claim 26 wherein the  
nucleic acid is as defined in claim 22 or claim 23.
28. A method as claimed in claim 26 or claim 27, wherein the expressed  
15 polypeptide product is subject to cleavage.
29. A pharmaceutical formulation comprising a polypeptide of any one of claims  
1 to 21, optionally together with a diluent, carrier or excipient.
- 20 30. A polypeptide as defined in any one of claims 1 to 21 for use in the  
treatment of disease.
31. The use of a polypeptide as defined in any one claims 1 to 21 in the  
manufacture of a medicament for the treatment of disease associated with  
25 protein/polypeptide structure.
32. A nucleic acid molecule as defined in claims 22 or claim 23 for use in the  
treatment of disease.

33. The use of nucleic acid molecule as defined in claim 22 or claim 23 in the manufacture of a medicament for the treatment of disease associated with protein/polypeptide structure.

- 5 34. A method of reconditioning a molecule preferably a protein comprising contacting said protein with a polypeptide of any one of claims 1 to 21.

35. A method as claimed in claim 34, wherein the protein is subjected to inactivation or denaturation prior to contacting with said polypeptide.

10

36. A method as claimed in claim 34 or claim 35, wherein the polypeptide is immobilised to a solid phase.

- 15 37. A method as claimed in claim 33 or claim 34, wherein the polypeptide is immobilised to a solid phase, preferably a chromatographic matrix, and the contacting of protein and polypeptide is carried out by applying the protein to the top of a bed of the matrix packed in a column and then eluting the polypeptide through the column.

- 20 38. Use of a polypeptide as claimed in any one of claims 1 to 21 for altering the structure of a molecule.

39. The use of claim 38, wherein the molecule is a protein or polypeptide and the alteration in structure is by folding unfolding or refolding.

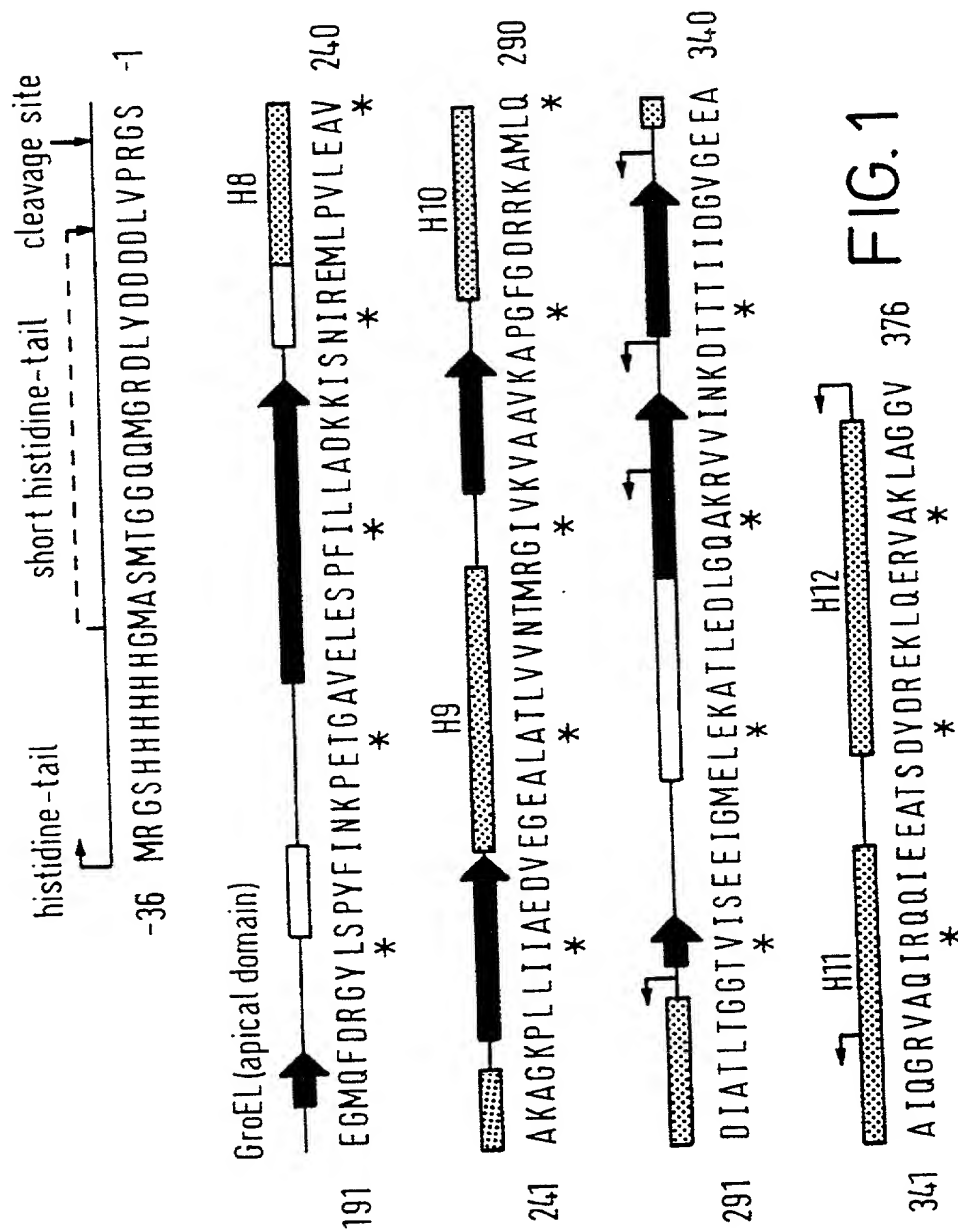
25

40. The use of claim 38 or claim 39, wherein the stoichiometry between the polypeptide and the molecule being altered is about 1:1.

41. Use of a polypeptide as defined in any one of claims 1 to 21 in the purification or increase in yield, specific activity or quality of biological molecules, preferably said polypeptide being attached to a support.
- 5 42. A kit for reconditioning or refolding a molecule, preferably a protein, comprising a polypeptide of any one of claims 1 to 21 immobilised to a solid phase and a container for holding said solid phase polypeptide.
43. Use of a polypeptide as defined in any one of claims 1 to 21 in the  
10 production of a protein or polypeptide by recombinant means, wherein the said polypeptide is co-expressed with the protein or polypeptide thereby to improve the yield or quality of the protein or polypeptide.
44. An antibody reactive against a polypeptide as defined in any one of claims 1  
15 to 21.
45. An antibody as claimed in claim 44 for use in the treatment of disease.
46. The use of an antibody as claimed in claim 44 in the manufacture of a  
20 medicament for the treatment of disease associated with protein/polypeptide structure.
47. A method of treating disease in which an effective amount of a polypeptide of any one of claims 1 to 21 is administered.  
25
48. A method of treating disease which comprises administering an effective amount of an inhibitor of the chaperone activity of a polypeptide of any one of claims 1 to 21.
- 30 49. A method as claimed in claim 48 wherein said inhibitor is an antibody.

50. A method of treating disease by gene therapy which utilises a construct encoding a polypeptide of any one of claims 1 to 20 or an antagonist thereof.

1/15



2 / 15

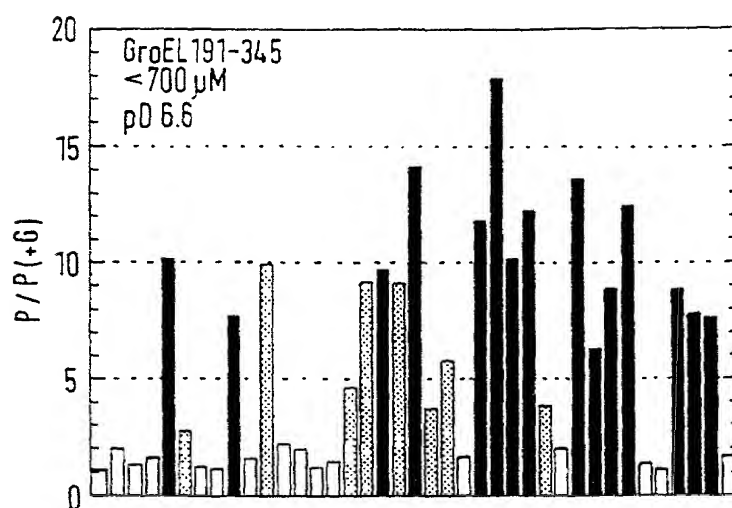


FIG. 2a

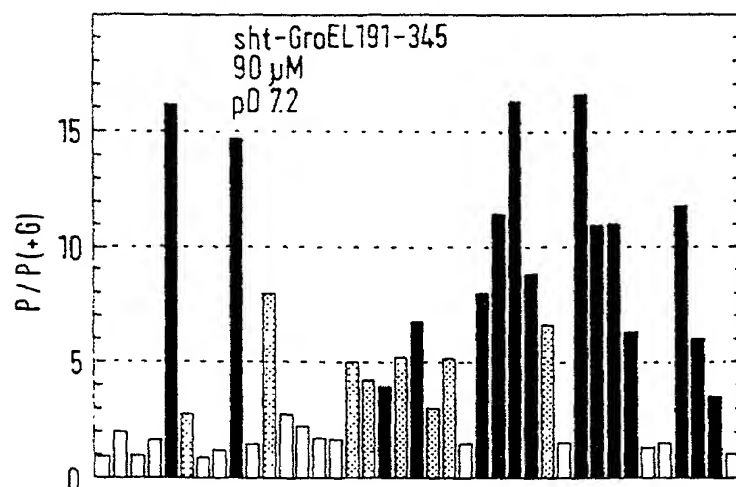


FIG. 2b

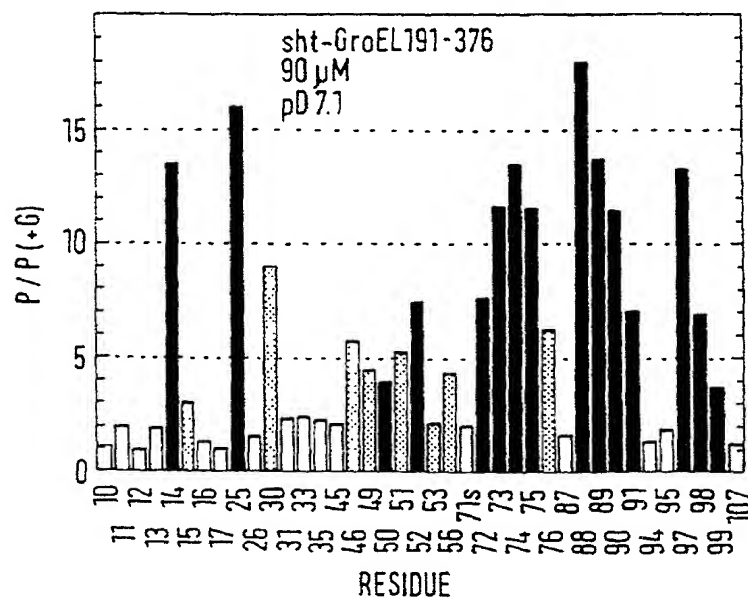


FIG. 2c

3 / 15

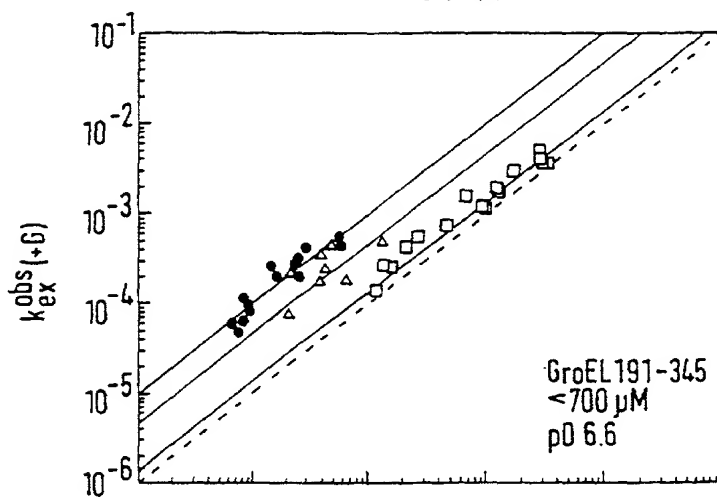


FIG. 2d

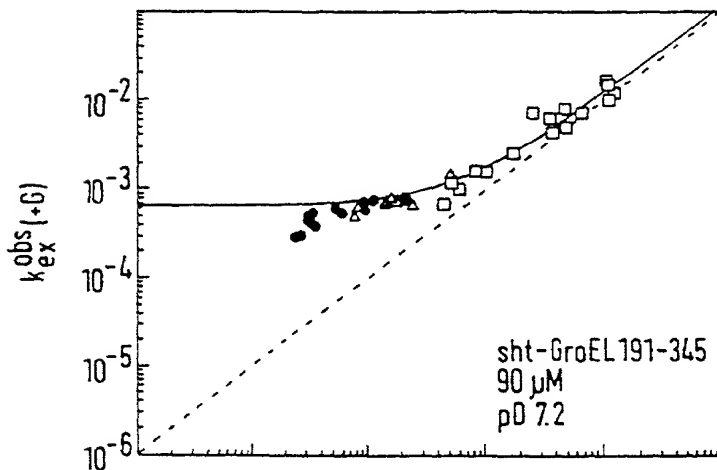


FIG. 2e

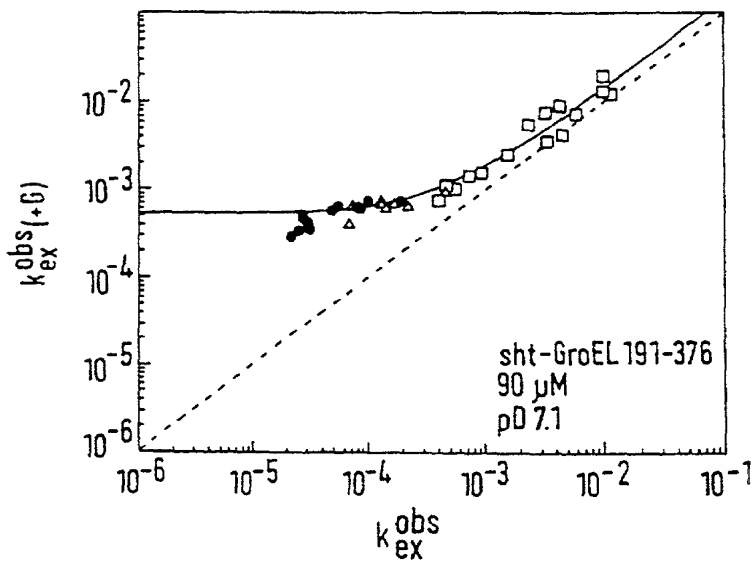


FIG. 2f



4 / 15

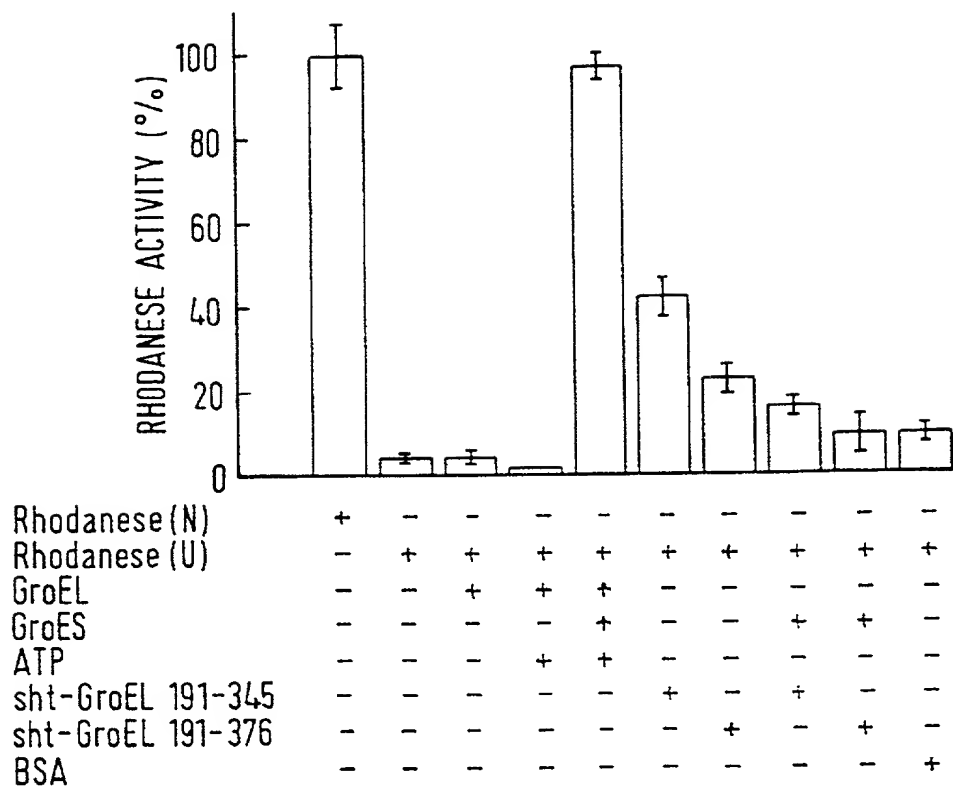
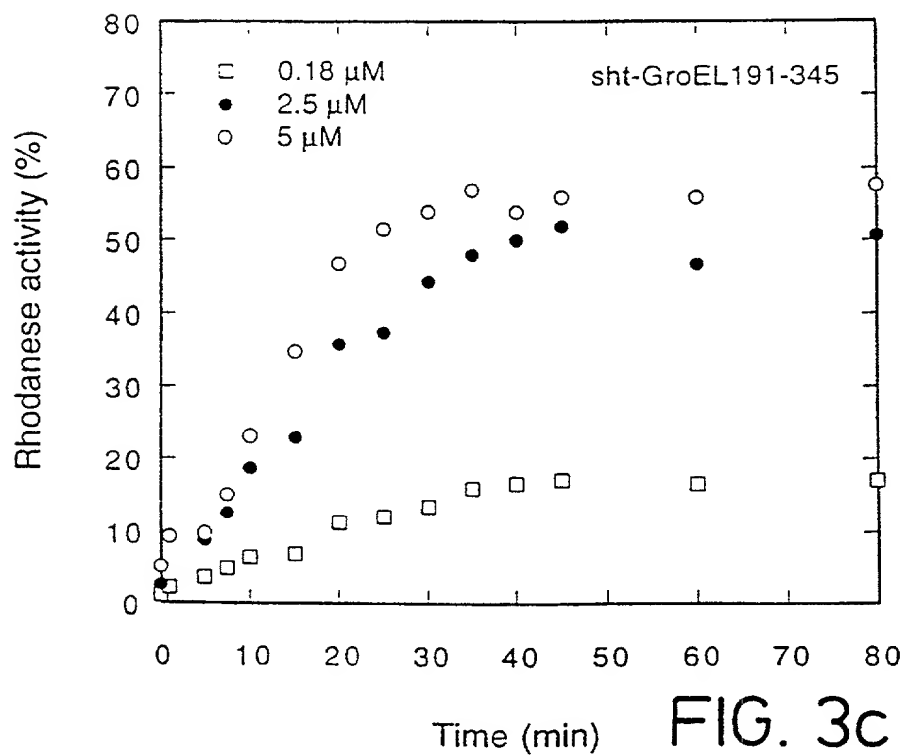
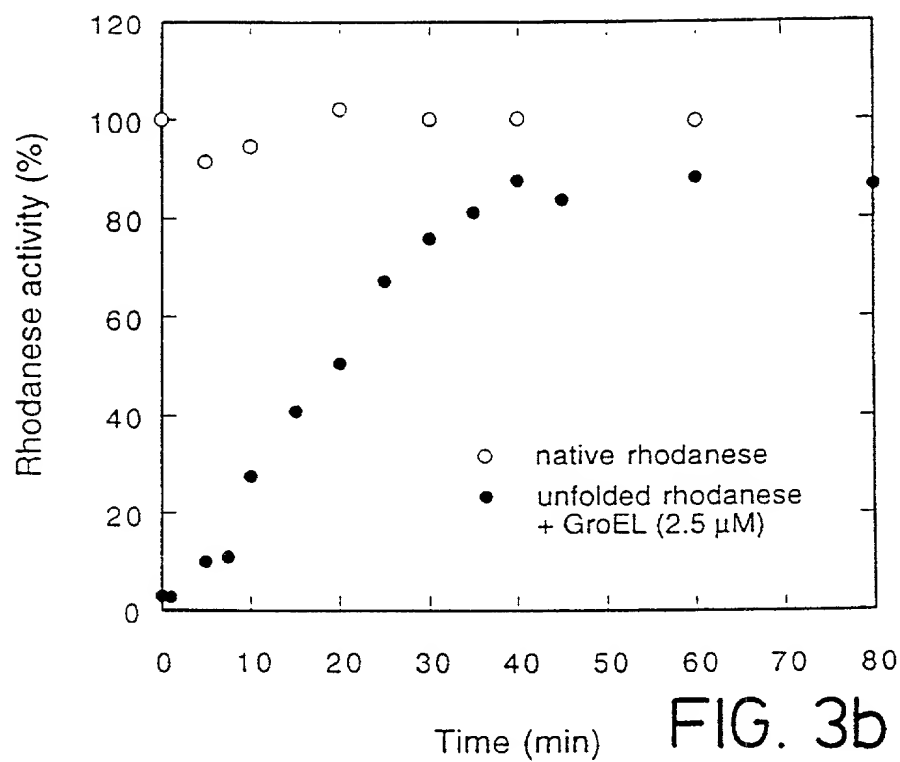
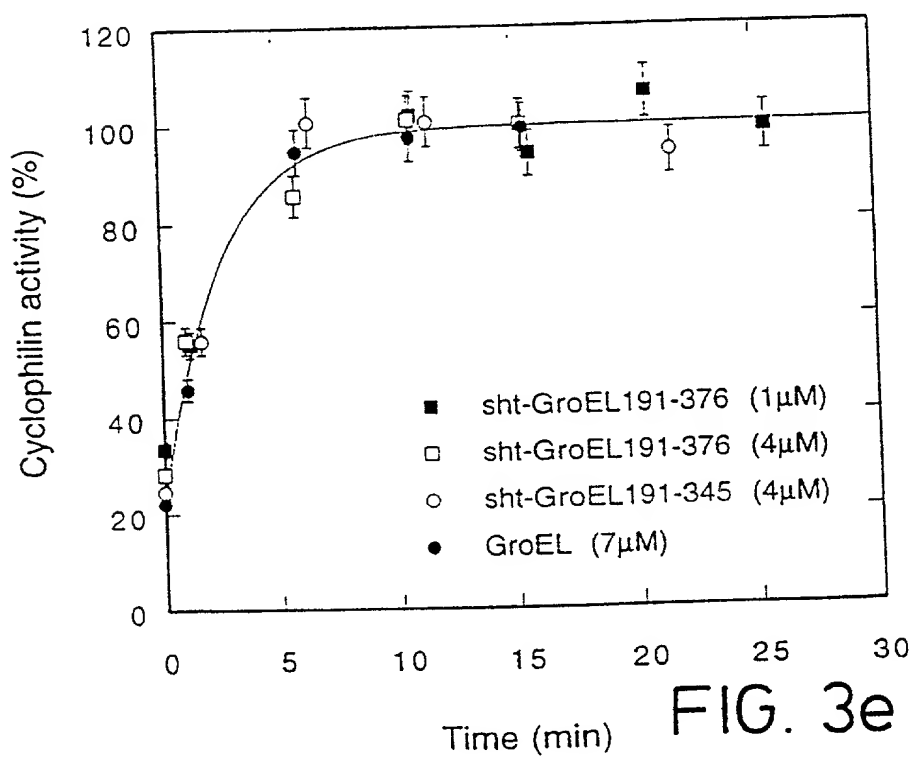
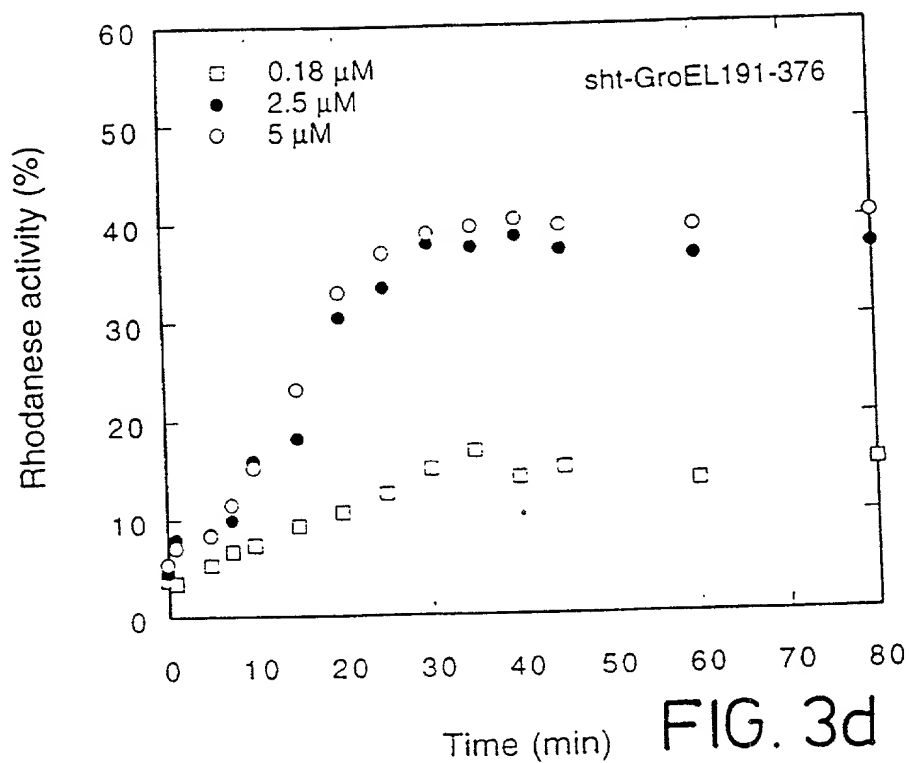


FIG. 3a

5 / 15



6/15



7/15

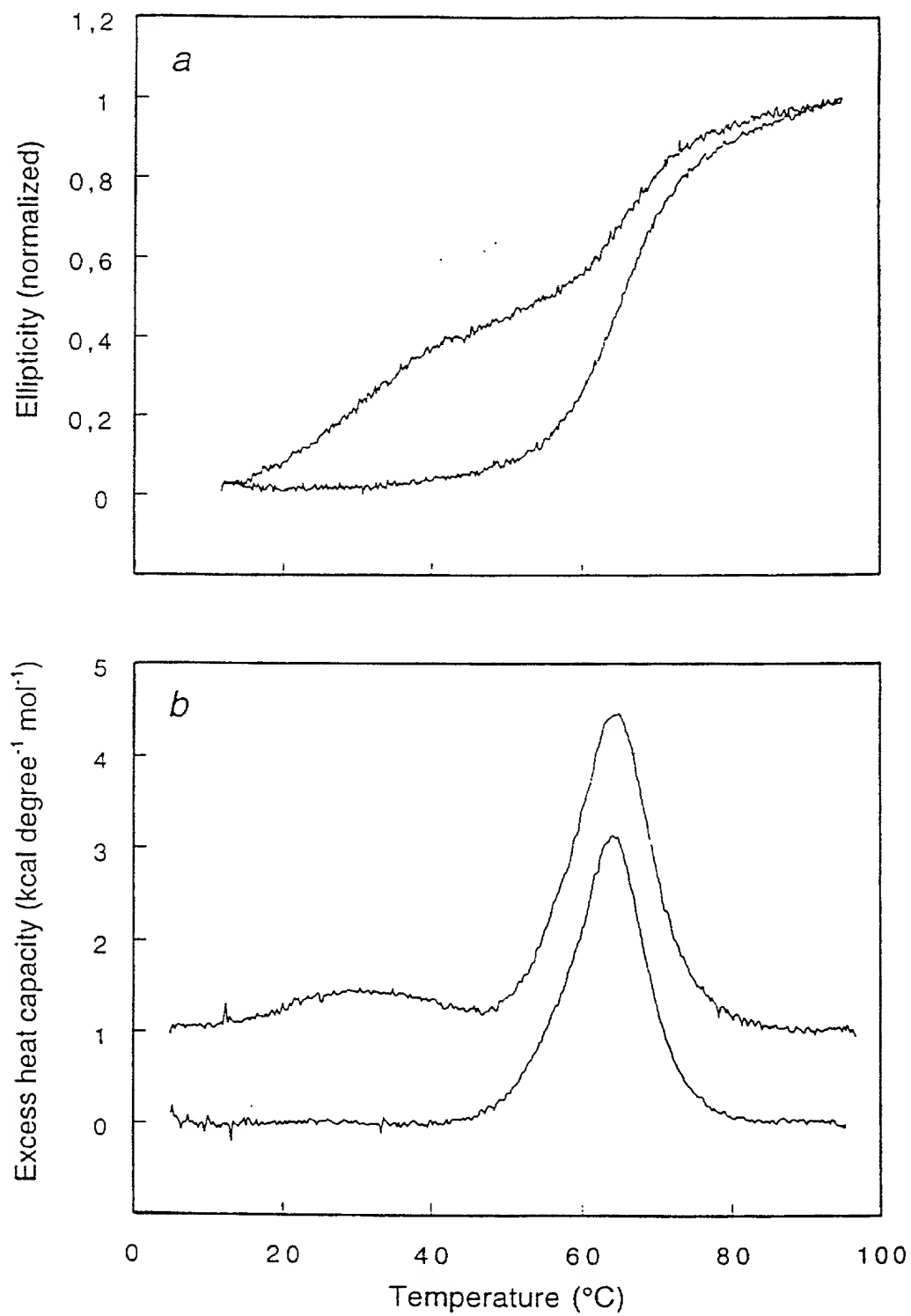


FIG. 4

8 / 15

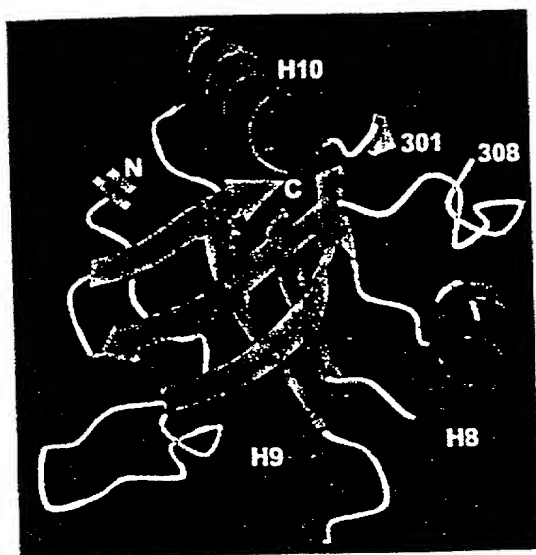


FIG. 5a

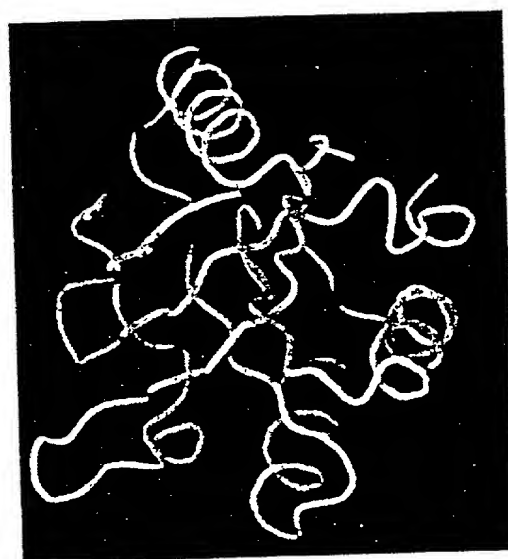


FIG. 5b

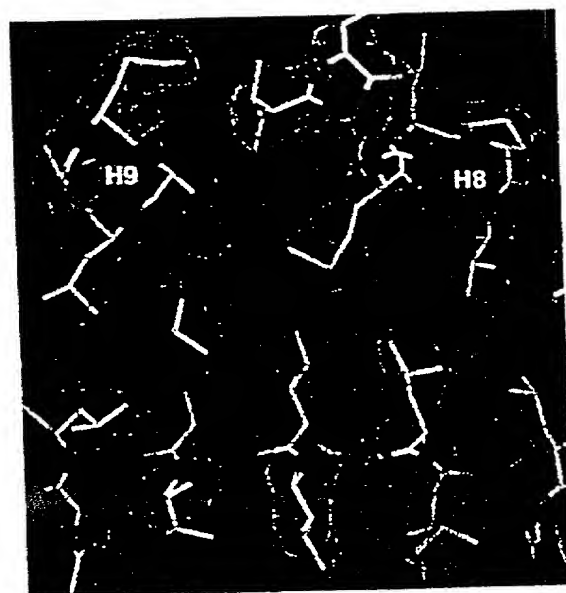


FIG. 5c

9 / 15

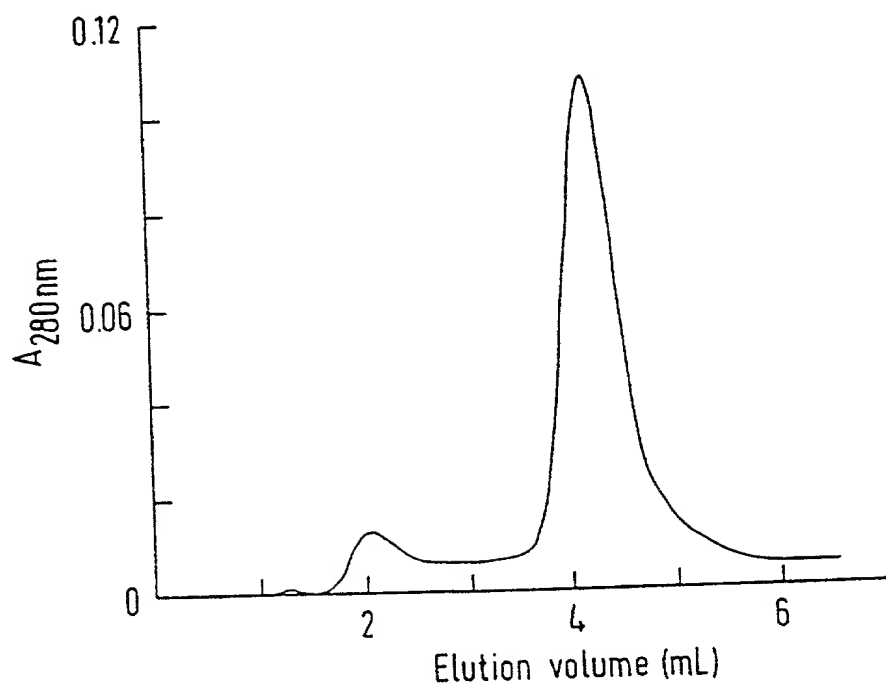


FIG. 6

10 / 15

AAKDVKFGND	ARVKMLRGVN	VLADAVKVTL	GPKGRNVVLD	KSFGAPTITK	DGVSVAREIE	1
LEDKFFENMGA	QMVKEVASKA	NDAAGDGT	ATVLAQAII	EGLKAVAAGM	NPMDLKRIGID	61
KAVTAAVEEL	KALSVPCSDS	KAIAQVGTIS	ANSDETVGKL	IAEAMDKVVK	EGVITVEDGT	121
GLQDELVDVE	GMQFDRGYLS	PYFINKPETG	AVELESPFIL	LADKKISNIR	EMLPVLEAVA	181
KAGKPLIIA	EDVEGEALAT	AVVNTIRGIV	KVAAVKAPGF	GDRRKAMLQD	IATLTGGTVI	241
SEEIGMELEK	ATLEDLGQAK	RVVINKDTT	IIDGVGEEAA	IQGRVAQIRQ	QIEEATSDYD	301
REKLQERVAK	LAGGVAVIKV	GAATEVEMKE	KKARVEDALH	ATRAAVEEGV	VAGGGVALIR	361
VASKLADLRG	QNEQNVGK	VALRAMEAPL	RQIVLNCGEE	PSVVANTVKG	GDCNYGYNA	421
TEEYGNMIDM	GILDPKVT	SALQYAAASVA	GLMITTECMV	TDLPKNDAAD	LGAAGGMGM	481
GGMGMM						541
						548

FIG. 7

11/15

	230	240	250	260	270
CH60_ECOLI_190	* x	x xx	*	x xx xxx	x xx
CH60_SALTI_190					*
S56371_191					
CH60_LEPIN_190					
S47530_191					
LPNHTPBG_190					
CH60_ACTAC_189					
JC4519_191					
CH60_BRUAB_191					
CH60_HAEIN_191					
CH60_CAUCR_190					
CH60_AMOPS_190					
CH60_HAEDU_191					
CH61_RHIME_191					
CH60_LEGMI_190					
CH60_YEREN_191					
CH63_BRAJA_190					
CH60_PORGI_191					
S52901_191					
S26423_191					
RSU373691_191					
CH62_BRAJA_190					
CH60_ACYPS_191					
CH63_RHIME_191					
YEPHSPCRP1_191					
CH60_BORPE_191					

FIG. 9(a)



12 / 15

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BRUGRO1_189      LQALLPVLEAV.....VQTSKPLLI IAEDVEGERLATLVVNKLRRGGL
CH60_PSEAE_191   IREMLPVLEAV.....AKAGRPLLI IAEDVEGEALATLVVNNMRGIV
CH60_BARBA_190   LQSLLPVLEAV.....AQSGKPLLI IAEDVEGEALATLVVNKLRRGGL
BAOB63A_191      LQSLLPVLEAV.....VQSGKPLLI IAEDVEGEALATLVVNKLRRGGL
CH60_BACST_189   IQEILPVEQV.....VQQGRPLLI IAEDVEGEALATLVVNKLRRGGL
CH60_BACSU_188   IQEILPVEQV.....VQQKPLLI IAEDVEGEALATLVVNKLRRGGL
CH60_AGRU_191    LQAMLPVLEAV.....VQTKPLVI IAEDVEGERLATLVVNKLRRGGL
B36917_191       LQAMLPVLEAV.....VQTKPLVI IAEDVEGERLATLVVNKLRRGGL
PAU17072_191     IREMLPVLEAV.....AKAGRPLLI IAEDVEGEALATLVVNNMRGIV
CH60_RHILV_191   LQSMPLPVLEAV.....VQTKPLLI IAEDVEGEALATLVVNKLRRGGL
CH61_STRCO_187   IADLLPVEQV.....QANASKPLLI IAEDLEGEALSTLVVNKIRGTF
CH60_COXBU_191   IRELIPLENV.....AKSGRPLLI IAEDIEGEALATLVVNNIRGVV
CH62_RHIME_191   LQAMIPLESV.....IQSGKPLLI IAEDVEGEALATLVVNKLRRGGL
PSEGR0ESL1_191   IREMLPVLEAV.....AKAGRPLLI IAEDVEGEALATLVVNNMRGIV
CH61_SYNY3_189   VQDLVPVEQV.....ARQKPLLI IAEDIEKEALATLVVNRLRGVL
CH60_CLOTM_189   IQDILPVEQV.....VQQKKLVI IAEDVEGEALATLVVNKLRRGGL
CH60_PSEPU_191   SRAAASATERA.....SAGRPLLI IAEDVEGEALATLVVNNMRGIV
CH60_SYNTP_190   VQDLVPVEQV.....ARAGRPLVI IAEDIEKEALATLVVNRLRGVL
CH60_GALSU_190   VQDILPVEQV.....AKTNKPLLI IAEDIEKEALATLVVNKLRRGIL
CH60_ZYMO_190    LQDILPVEQV.....VQSGRPLLI IAEDIEGEALATLVVNKLRRGGL
JC2564_191       LQDILPVEQV.....VQSGRPLLI IAEDIEGEALATLVVNKLRRGGL
CH60_CHRVI_191   IRDILPVLEGV.....AKAGKPLLI IAEDVEGEALATLVVNTIRGIV
CH60_MYCTU_189   VKDILPVEKV.....IGAGKPLLI IAEDVEGEALSTLVVNKIRGTF
CH60_NEIME_191   IRDILPVLEKV.....AKASRPLLI IAENVEGEALATLVVNNIRGIL
CH60_TREPA_189   MKDILPVEKV.....AQTKRPLLI IAEDVEGEALATLVVNSLRGTL
CH60_HELPY_190   MKDILPVEKV.....MKEGKPLLI IAEDIEGEALATLVVNKLRRGVL
CH60_NEIGO_191   IRDILPVLEQV.....AKASRPLLI IAEDVEGEALATLVVNNIRGVL
CH61_CUCMA_222   INAVVKVLELA.....LKKORPLLI VSEDVESEALATLVVNKLRRGIL
CH60_MYCPA_189   VKDILPVEKV.....IQAGKPLLI IAEDVEGEALSTLVVNKIRGTF
MPU15989_230     VKDILPVEKV.....IQAGKPLLI IAEDVEGEALSTLVVNKIRGTF

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FIG. 9(b)

13/15

S26582\_224 MHAVVKVLEMA.....LKKQKPLLIIVAEDEVSEALGTLLINKLRAGI  
 S40247\_191 IRDLLPVLEQV.....AKASRPLLIIVAEDEVSEALGTLLVNNIRGVL  
 CH60\_CLOAB\_189 IQEILPPEQI.....VQGGKLLIIAEDVEGEALATLVNKLRTGF  
 CH60\_NEIFL\_191 IRDLLPVLEQV.....AKASRPLLIIVAEDEVSEALGTLLVNNIRGIL  
 CH60\_LEGPN\_190 IREMLSVLEGV.....AKSGRPLLIIVAEDEVSEALGTLLVNNMRGIV  
 CH62\_CUCMA\_222 INSVVKVLELA.....LKRQRPILLIVSEDEVSEALGTLLINKLRAGI  
 CHTGROESL1\_191 IKDFLPVLQOV.....AESGRPLLIIVAEDEVSEALGTLLVNNIRGGF  
 S40172\_64 IKDFLPVLQOV.....AESGRPLLIIVAEDEVSEALGTLLVNNIRLRAF  
 SYOGROEL2\_189 IQDLIPTLERV.....ARSGRPLVIIVAEDEVSEALGTLLVNNKLRGVL  
 CH60\_CHLPS\_191 IKDFLPVLQOV.....AESGRPLLIIVAEDEVSEALGTLLVNNRLRAGF  
 CH62\_STRAL\_188 VKDLLPPEKV.....MQSGKPLLIIVAEDEVSEALSTLVNKLRTGF  
 CH62\_MYCLE\_189 VKDLLPPEKV.....IQAGKSLLIIIVAEDEVSEALSTLVNKLRTGF  
 MSGANTM\_236 VKDLLPPEKV.....IQAGKSLLIIIVAEDEVSEALSTLVNKLRTGF  
 CH60\_BORBU\_190 IKELLPVLEKV.....LGTNKPILLIIIVAEDEVSEALGTLLINKLRAGI  
 S26583\_224 MHAVVKVLEMA.....LKKQKPLLIIVAEDEVSEALGTLLINKLRAGI  
 A49209\_190 IKELLPVLEKV.....LGTNKPILLIIIVAEDEVSEALGTLLINKLRAGI  
 MZECPN60B\_224 MHAVVKVLEMA.....LKKQKPLLIIVAEDEVSEALGTLLINKLRAGI  
 CH60\_THEP3\_189 IQELLPALEQV.....VQQRPLLIIVAEDEVSEALGTLLVNNKLRTGF  
 CH60\_STAEP\_188 FQDILPPEQV.....VQASRPILIVAEDEVSEALGTLLVNNKLRTGF  
 CH60\_LACLA\_189 IQEILPPEQI.....LKTNRPLLIIVADDVDGEALPTLVNKLRTGF  
 CH61\_STRAL\_188 IQDLLPPEKVI.....QAGGSKPLLIIVAEDEVSEALSTLVNKLRTGF  
 CH60\_CHLPN\_191 IKDFLPVLQOV.....AESGRPLLIIVAEDEVSEALGTLLVNNRLRAGF  
 MZECFN60A\_224 MHAVVKVLEMA.....LKKQKPLLIIVAEDEVSEALGTLLINKLRAGI  
 HECHSPAB1\_190 MKDILPPEKT.....MKEGKPLLIIVAEDEVSEALGTLLVNNKLRTGF  
 CH60\_ARATH\_221 INSIVKVLELA.....LKRQRPILLIVSEDEVSEALGTLLINKLRAGI  
 CH60\_MAIZE\_224 MHAVVKVLEMA.....LKKQKPLLIIVAEDEVSEALGTLLINKLRAGI  
 CH60\_CHLTR\_190 IKDFLPVLQOV.....AESGRPLLIIVAEDEVSEALGTLLVNNIRGGF  
 CH60\_STAAU\_189 FQDILPPEQV.....VQSNRPILIVAEDEVSEALGTLLVNNMRGTF  
 CH60\_CLOPE\_189 IQDLLPPEQI.....VQAGKLLIIIVAEDEVSEALGTLLVNNKLRTGF  
 HS60\_YEAST\_212 IQDILPALEIS.....NQSRPPLLIIVAEDEVSEALGTLLVNNKLRTGF  
 CH60\_PYRSA\_217 VQELVPIHLELI.....SKTSRPLLIIVAEDEVSEALGTLLVNNKLRTGF

FIG. 9(c)

14 / 15

CH60_EHRCH_191	IQIPLILENV.....	ARSGRPLLI	IAEDVEGEALSTLVNKLRGGL
CHTGR0E1_191	IKDFLPVQQV.....	AESGRPLLI	IAEDIVGEALATLVVNRIRGGF
CH60_THETH_188	VRELLPILEQV.....	AQTGKPLLI	IAEDVEGEALATLVVNKLRGTL
TAU294831_189	VRELLPILEQV.....	RQTGKPLLI	IAEDVEGEALATLVVNKLRGTL
CH60_RICTS_190	VQPLVPVLEAV.....	AHTGKPLVLI	IAEDVDGEALTALILNNLKGS
SYCCPNC_189	IAELVPVLEAV.....	ARAGRPLLI	IAEDIEGEALATLVVNKARGVL
CPU308211_190	VQDLVPVLEQV.....	ARARKPLFI	IAEDIEKEALATLVVNKLKRGNL
CH61_MYCLE_189	LPELLPMLEKV.....	TESGKPLLI	IAEDLEGEALATLVVNSIRKTL
PSU21139_239	ARDLINILEDA.....	IRSGFPIVI	IAEDIEQEALATLVVNKLKRGSL
CH60_COWRU_191	IQPLLPILENI.....	ARSGRPLLI	IAEDVEGEALSTLVNKLKRGGL
RUBB_BRANA_245	ARDLVGVLEDA.....	IRGGYPILI	IAEDIEQEALATLVVNKLKRGTL
SCCPN60_144	ARDLINVLEEA.....	IRGQYPILI	IAEDIEQEALATLVVNKLKRGSL
CH60_EHRI_153	IQPMVPLETI.....	VRNRAVVI	IAEDVEGEALSTLVLSKMRGSL
RUBB_ARATH_245	ARDLVGVLEDA.....	IRGGYPILI	IAEDIEQEALATLVVNKLKRGTL
ATU49357_235	IKDIIPILEKT.....	TQLRAPLLI	IAEDVTGEALATLVVNKLKRGVL
RUB1_BRANA_195	IKDIIPILEKT.....	TQLRAPLLI	IAEDVTGEALATLVVNKLKRGVL
CH62_SYNY3_189	IAELVPVLEAV.....	ARAGRPLLI	IAEDIEGEALATLVVNKARGVL
RUBA_RICCO_178	IKDIIPILEKT.....	TQLRAPLLI	IAEDVTGEALATLVVNMARGIL
CH60_ODOSI_190	VQDILLPILEQI.....	TKTKRPLLI	IAEDVEKEALATLILNKLKRGIV
PSU21105_236	IKDIIPILEKT.....	TQLRAPLLI	ISEDITGEALATLVVNKLKRGIL
CH60_BRANA_224	LNSMVKVLELA.....	LKSQSRLLI	IVAADLES DALAVLILNKLKLAGI
PMSARG2_105	IADLVPVLETV.....	QKSSPLVLI	IAEEVDGEALATLVVKNRNGVL
RUB2_BRANA_234	IKDIIPILEKT.....	TQLRAPLLI	IAEDVTGEALATLVVNKLKRGVL
CRECPN1A_75	ARDIITILESA.....	IRGNYPPLI	IAEEVEQEALATLVVNKLKRGTL
P60_CRIGR_215	VQSIVPALEIA.....	NAHRKPLVI	IAEDVDGEALSTLVNRLKVGVL
CRECPN1B_224	IRDIIPILEQV.....	TRLNAPLLI	IAEDVSGEALATLVVNKLKRGVL
RUBA_WHEAT_191	IKDIIPILEQV.....	TQLRCPLFI	IAEDITGEALATLVVNAIRKTL
B47292_189	LPDLLPLEKV.....	AGTGKPLLI	IAEDVEGEALATLVVNAIRKTL
CELHSP60CP_206	VQDIVPALELA.....	NKLRRPLVI	IAEDVDGEALTTLVNLKVGVL
P60_HUMAN_215	IQSIVPALEIA.....	NAHRKPLVI	IAEDVDGEALSTLVNRLKVGVL
P60_MOUSE_215	VQSIVPALEIA.....	NAHRKPLVI	IAEDVDGEALSTLVNRLKVGVL

FIG. 9(d)

15/ 15

P60 RAT 215	VQSIVPALEIA.....	NAHRKPLVIAEDVDGEALSTLVNRLKVGIL
A4131 215	VQSIVPALEIA.....	NAHRKPLVIAEDVDGEALSTLVNRLKVGIL
MMHSP60A 197	VQSIVPALEIA.....	NAHRKPLVIAEDVDGEALSTLVNRLKVGIL
CH63 HELVI 218	ASQVVPALALA.....	NSQKKPLVIAEDYDGEPLSVLVNKLKIGL
EGHSP60GN 205	IQQILPVLEHI.....	AKANRPLVIAEDVDSEALATLIINKINGKL
HS60 SCHPO 222	VQDILPSLELA.....	AQQRPLVIAEDVDGEALAACILNKLRGQL
S61295 198	IHTILPALNHV.....	VGTGRPLLIADDDVESEALTMI FNKLQGKL
TRBMTHSP 198	IHTILPVLNHV.....	VRSGRPLLIADDDVESEALTMI FNKLQGKL
ENHCPN60P 142	SQSVVPILLELC.....	LKRKRPLVVISDTIDGDALTTLVLNKLRLGL
CH60 PLAFG 257	IQSILRILEIFAKNKQPLCIIADDFSNVLTLLINKLKGAIKVLCIVTNSKYISADVGLDLNNLHNNMSSFD	
CH60 MYCGE 189	IKEILPLLEGS.....	VENGNPILLIVAPDFAEEVVTTLAVNKLRTI
HTOHSP60X 228	VQDIIPALEAS.....	TTLRRPLVIAEDIEGEALAVCILNKLRRPT

FIG. 9(e)

**DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY**  
(Includes reference to PCT International Applications)

FROMMER LAWRENCE & HAUG, LLP  
File No.: 674508-2001

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and joint inventor (if plural, names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention ENTITLED:

**CHAPERONE FRAGMENTS**

the specification of which:

- is attached hereto
- X was filed on March 25, 1999 as:
- X United States Application Serial No. \_\_\_\_\_ as a continuation-in-part of  
PCT Application No. PCT/GB97/02652
- X with amendments through DATE EVEN HEREWITH (if applicable, give details).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119 (a) - (d) or § 365 (b) of any foreign application(s) for patent or inventor's certificate, or § 365 (a) of any PCT International application(s) designating at least one country other than the United State of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT International applications designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign/PCT Application(s) [list additional applications on separate page]:

Priority Claimed:

<u>Country (or PCT)</u>	<u>Application Number:</u>	<u>Filed (Day/Month/Year)</u>	<u>Yes</u>	<u>No</u>
PCT	GB97/02652	26 SEPTEMBER 1997	X	
PCT	GB96/02980	3 DECEMBER 1996	X	
U.K.	9620243.7	26 SEPTEMBER 1996	X	

(GREAT BRITAIN, GB)

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

I hereby claim the benefit under Title 35, United States Code § 120 of any United States application(s) or § 365 (c) of any PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior United States or PCT International application(s) in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Prior U.S. (or U.S.-designating PCT) Application(s) [list additional applications on separate page]:

U.S. Serial No.: Filed (Day/Month/Year) PCT Application No. Status (patented, pending, abandoned)

PCT/GB97/02652      26 SEPTEMBER 1997  
PCT/GB96/02980      3 DECEMBER 1996

PENDING

I hereby appoint Thomas J. Kowalski, Registration No. 32,147 and FROMMER LAWRENCE & HAUG, LLP or their duly appointed associates, my attorneys or agents, with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to file continuation and divisional applications thereof, to receive the Patent, and to transact all business in the Patent and Trademark Office and in the Courts in connection therewith, and to insert the Serial Number of the application in the space provided above, and specify that all communications about the application are to be directed to the following correspondence address:

Thomas J. Kowalski, Esq.  
c/o FROMMER LAWRENCE &  
HAUG, LLP  
745 Fifth Avenue  
New York, NY 10151  
FAX (212) 588-0500

Direct all telephone calls to: (212) 588-0800  
to the attention of:  
Thomas J. Kowalski

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

INVENTOR(S):

Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Full name of first inventor: Alan Roy FERSHT  
Residence: : Lensfield Road, Cambridge CB2 1EW, Great Britain  
Citizenship: BRITISH

Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Full name of second inventor: Ralph ZAHN  
Residence: Lensfield Road, Cambridge CB2 1EW, Great Britain  
Citizenship: BRITISH

Signature: \_\_\_\_\_ Date: \_\_\_\_\_

Full name of third inventor: Myriam Marlenne ALTAMIRANO  
Residence: 302 Hills Road, Cambridge CB2 2QG, Great Britain  
Citizenship: MEXICAN

Post Office Address(es) of inventors [if different from residence]:

NOTE: In order to qualify for reduced fees available to Small Entities, each inventor and any other individual or entity having rights to the invention must also sign an appropriate separate "Verified Statement (Declaration) Claiming [or Supporting a Claim by Another for] Small Entity Status" form [e.g. for Independent Inventor, Small Business Concern, Nonprofit Organization, Individual Non-Inventor].